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FREQUENCY OF EXTENDED DIAPAUSE IN NEBRASKA POPULATIONS OF *DIABROTICA BARBERI* SMITH AND LAWRENCE.

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FREQUENCY OF EXTENDED DIAPAUSE IN NEBRASKA POPULATIONS OF *DIABROTICA BARBERI* SMITH AND LAWRENCE.

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FREQUENCY OF EXTENDED DIAPAUSE IN NEBRASKA POPULATIONS OF *DIABROTICA BARBERI* SMITH AND LAWRENCE.

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This study was conducted to measure the frequency of extended diapause in populations of the northern corn rootworm, *Diabrotica barberi* Smith and Lawrence from eastern Nebraska. Adult *D. barberi* collections were made during late summer 2008 and 2009 from eight sites each year (seven sites were consistent over years). Eggs were obtained from 12-20 females per site and were held on moist soil under appropriate temperature profiles to facilitate egg survival, diapause development, and diapause termination within and among years. Percentage egg hatch was recorded after the first and second year for the 2008 collection and after the first year for the 2009 collection. Additional extended diapause expression was estimated for the 2008 collection by counting remaining live eggs after the second year egg hatch was completed. This data was also used to estimate each site's maximum egg viability. Results collectively indicate that the extended diapause trait occurred in all eastern Nebraska populations; however, significant variation in the frequency of extended diapause was observed within and among *D. barberi* populations. Geographically, the highest incidence of extended diapause was found in a north-south transect in eastern counties of Nebraska that parallel the Missouri River. In general, the frequency of extended diapause was lower in sites found west of the north-south transect. . This study contributes to the database needed to develop appropriate *D. barberi* management strategies in Nebraska. Data can be used to pinpoint areas of

Nebraska that may have the greatest risk of economic injury in first-year corn when *D. barberi* densities are high. Extended diapause in Nebraska *D.barberi* populations may also be a positive attribute from a resistance management perspective. Diapausing individuals will provide a natural refuge which would complement any structured refuge that may be in place when transgenic plants are used to manage corn rootworms.

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES

FIGURE **PAGE**

LIST OF TABLES

Chapter 2

. Appendix 1.

CHAPTER 1

INTRODUCTION / LITERATURE REVIEW

Taxonomic History

Diabrotica Chevrolat (Coleoptera: Chrysomelidae: Galerucinae), a largely Neotropical genus, is comprised of 354 reported species (Wilcox 1972, Krysan and Smith 1987, Krysan 1999, Branson and Krysan1981). Three species groups make up the genus *Diabrotic*: *fucata*, *signifera* and *virgifera* (Wilcox 1972). The *fucata* group includes more than 300 species, and ranges from South America to the United States in North America. The *signifier* group includes 11 species and is only found in Central and South America, while the 35 species in the *virgifera* group are found primarily in North and Central America (Krysan and Smith 1987, Cabrera Walsh 2003). Five species of the *virgifera* group occur in the United States, and *Diabrotica virgifera virgifera* Le Conte (western corn rootworm) and *Diabrotica barberi* Smith and Lawrence (northern corn rootworm) are key pests of corn, *Zea mays L.,* in the Corn Belt (Krysan 1986). These two species are commonly referred to as corn rootworms because the larvae primarily feed on the roots of corn (Branson and Krysan 1981). Corn rootworms are responsible for an estimated $1 billion annually in control costs and yield losses in the United States (Metcalf 1986, Sappington et al. 2006).

In the 1870s, a rootworm species was documented feeding on continuous corn, in Illinois and Missouri, which morphologically was very similar to *Diabrotica longicornis* (Say) that had been originally collected from wild cucurbits in Colorado (Say 1824).

Because of the similarity, the rootworm found on corn was also called *D. longicornis* (Webster 1913, Krysan et al. 1983). Eventually, *D. longicornis* was formally split into two subspecies by Smith and Lawrence (1967) who separated the corn pest, *D. longicornis barberi,* and the non-corn pest *D. longicornis longicornis*. In 1983, Krysan et al. elevated *D. l. barberi* to species rank based on differences in behavior (e.g., pheromone response, unidirectional hybridization, and habitat choice), range, and color between the two beetles. *D. longicornis* occurs from Nebraska to Texas and southwest to Arizona and Chihuahua, Mexico. *D. barberi* can be found from Oklahoma to North Dakota and east to Ontario, Canada, and the northeastern United States. However, there is an overlap zone in which both taxa can be found. This overlap occurs in eastern Nebraska, eastern Kansas, and northern Oklahoma (Krysan and Smith 1987).

Biology

Diabrotica. barberi is univoltine; initial egg hatch occurs in late May to early June in the U.S. Corn Belt with adult emergence often in July and August (Metcalf and Metcalf 1992). Oviposition occurs in corn during mid-late summer. Eggs are laid in soil at the base of corn plants in non-irrigated fields and can also be found between rows in irrigated fields. Eggs are primarily found to soil depths of 7.6-23 cm but may be displaced due to the effects of tillage (Patel and Apple 1967, Chiang 1973). Eggs enter an obligatory resting stage, known as diapause, which allows them to survive the winter (Krysan 1999). The eggs of *D. barberi* enter diapause at a specific embryonic stage. At this time the embryo is segmented with clearly defined cephalic lobes (Krysan 1982). In order to complete diapause development most eggs need to be exposed to a cold period

(Krysan 1993). The developmental rate of the egg post-diapause is dependent on the number of heat units accumulated over time. Experiments have shown that the developmental threshold for *D. barberi* eggs is around ca. 11.11°C (Chaing and Sisson 1968, Apple et. al. 1971, Wilde et. al. 1972).

A proportion of some populations exhibit extended diapause which enables *D. barberi* to remain in the egg stage for more than one winter (Ostlie 1987). Extended egg diapause in *D. barberi* was first reported by Chiang (1965) in Minnesota but the frequency was only 0.3% of the population. Over time, the frequency of extended diapause has increased to 40-50% in some areas (Krysan et al. 1984, Krysan et al. 1986, Levine et al. 1992). Additionally, diapause for up to four years has been documented (Levine et al. 1992, Campbell 2009).

After eclosion from the egg, *D. barberi* larval development progresses through three instars, all of which remain in the soil feeding on the roots of corn (Hammack et al. 2003). Although corn is the most preferred larval host (Branson and Ortman 1971, Branson and Ortman 1967), it has been shown that *D. barberi* larvae are capable of developing on several different grass species. Some grass species have been examined in lab or greenhouse experiments for their potential as alternate hosts for *D. barberi*. Larvae were shown to complete development on pubescent wheatgrass, tall wheatgrass, weeping lovegrass, slender wheatgrass, and proso millet (Branson andOrtman 1971, Branson andOrtman 1967, Oyediran et al. 2004, Oyediran et al. 2008). However, to date, *D. barberi* has not been found to utilize these grasses as larval hosts in the field.

After third instar larvae have completed development, they create earthen cells in which to pupate. The pupae are often found in the top 5 cm of the soil profile but have been observed at soil depths of up to 23cm (Chiang 1973). When beetles emerge from the soil they start to feed on the reproductive parts of the corn plant, such as the silks and pollen of the plant (Cinereski and Chiang 1968). Once corn plants have completed pollination some beetles move on to find other sources of food (Cinereski and Chiang 1968, Campbell and Meinke 2006). This may include pollinating plants nearby, such as chrysanthemums, alfalfa, red clover, squash, pumpkin, sunflowers, and thistles (Forbes 1883). However, studies have shown that even after corn becomes a suboptimal adult food source, many mated *D. barberi* females return to corn to oviposit (Cinereski and Chiang 1968, Siegfried and Mullin 1990, Boetel et al. 1992).

D. barberi Population Variation

Diabrotica barberi exhibits relatively high levels of genetic diversity and variation of traits, especially within populations across the United States. This has been demonstrated in studies conducted over relatively small and large geographic scales (McDonald et. al. 1985, Krysan and Sutter 1986, Golden 1990, Krafsur et. al. 1993, Clark et al. 2001, Roehrdanz et. al. 2003, Campbell et al. "in press"). On a large scale, both McDonald et al. (1985), who analyzed variation of seven polymorphic allozyme loci, and Roehrdanz et al. (2003), who focused on genetic variation of mtDNA and the ITS1 gene, found that western *D. barberi* populations exhibited different genetic patterns than eastern populations across the Corn Belt. Introgression between *D. barberi* and *D. longicornis* has been suggested as one possible reason for substantial genetic variability

of *D. barberi* in the western Corn Belt (McDonald et al. 1985, Campbell et al. 'in press'). On a more local scale, several studies have documented that significant genetic differences can occur among *D. barberi* populations that are in close geographic proximity. Examples include Aldrin susceptibility (Ball and Weekman 1963, Krysan and Sutter 1986), amplified fragment length polymorphism (AFLP) variation (Campbell et al. 'in press'), and polymorphic allozyme loci (Krafsur et. al. 1993). It has been suggested that this variation could be because of the limited flight activity of *D. barberi,* which may lead to partially isolated breeding populations (McDonald et. al. 1985, Krafsur 1993). *D. barberi* typically moves outside of corn fields in order to find new sources of food (Cinereski and Chiang 1968, Naranjo and Sawyer 1988). However, flight studies have shown that *D. barberi* typically only takes short trivial flights and unlike the western corn rootworm, does not undertake long range migratory flights (Naranjo 1990).

Economic Importance

The majority of economic damage caused by *D. barberi* comes from the larval feeding on the roots of corn (Chaing 1973). This root damage can adversely affect corn development and cause corn plants to lodge (fall over) which collectively may reduce grain yield (Chiang 1973, Levine and Oloumi-Sadeghi 1991). Historically this has been a problem in continuous corn in parts of the U.S. Corn Belt (Levine et al. 1992).

Traditionally, crop rotation from a larval host to non-host crop (e.g., non-grass host, annual corn-soybean rotation) became an effective way to manage *D. barberi* in corn. However, significant corn rootworm damage to first-year corn in annual rotation with soybean has periodically occurred over the last 25 years in Minnesota, Iowa, and

South Dakota (Ostlie 1987). Research has documented that this phenomenon has been caused by a significant proportion of *D. barberi* populations in affected areas exhibiting extended diapause (Krysan et al. 1986). It is possible that the selection pressure on *D. barberi* created by crop rotation has led to increases in the frequency of extended diapause in some populations. Those that exhibit extended diapause would survive and therefore pass on the trait while those that do not would die when they hatch out on an unsuitable host (Ostlie 1987). However, the genetic basis of extended diapause in *D. barberi* has not been determined.

Pest Status in Nebraska

Over the last decade, northern corn rootworm densities and reports of rootworm injury in first year corn have been increasing in eastern Nebraska (Hunt et al. 2007). The reason is unclear but a series of mild winters which may have increased overwintering survival and/or increasing frequencies of *D. barberi* extended diapause may be contributing factors. A 2006-2007 survey of *D. barberi* emergence patterns in first-year corn fields revealed that population densities are highly variable in eastern Nebraska, but in general are much higher in northeast and east-central areas than locations farther south (Hunt et al. 2007, Meinke et al. unpub.). The presence of *D. barberi* extended diapause has been confirmed and characterized in a population from Saunders County, Nebraska (Campbell 2009), but the frequency of extended diapause has not been evaluated in other *D. barberi* populations around the state.

D. barberi* vs. *D. longicornis

It has been shown that *D. barberi* and *D. longicornis* are capable of hybridizing in laboratory experiments (Golden 1990, Campbell and Meinke 2010) and genetic analysis supports introgression between the species in the field (Campbell et al. "in press"). Research has shown that hybrids of a *D. longicornis* female and a *D. barberi* male were as viable as individuals of both parental species (Campbell and Meinke 2010).

It is well established that *D. barberi* prefers corn as a larval and adult food source (Branson and Ortman 1971, Branson and Ortman 1967, Naranjo 1994), but little is known about the major food hosts of *D. longicornis*. *Diabrotica longicornis* adults have been found in association with prairie ecosystems where perennial grasses are present. It is possible that their larvae feed on the roots of these grasses, but it has not been experimentally documented. In the laboratory, it is possible to rear *D. longicornis* on the roots of corn (Golden and Meinke 1991); however, larvae have never been found in cornfields and adults are rarely found in commercial corn (Branson and Krysan 1981). This is a major distinction between these two species. Several possible reasons for this difference could be variation in ovipositional and host habitat preferences among species. Part of this may involve the relative attractiveness of corn volatiles to each species. Volatiles from corn such as geranylacetone and (+)-a-terpineol have been found to be attractive to *D. barberi* beetles in field capture trials (Hammack 1997, Hammack 1996). However, it is unknown how *D. longicornis* or *D. barberi* x *D. longicornis* hybrids respond behaviorally to volatiles of corn. Also, with the possibility of hybrids (and various backcrosses) in the area of sympatry, it is possible that populations of *D. barberi*

found in northern Nebraska could be behaviorally different from those found in southern Nebraska. This may differentially affect *D. barberi* management across the region (Campbell and Meinke 2010).

Goal

The overall goal of this study is to gain a better understanding of the biology and behavior within and among populations of *D. barberi* in eastern Nebraska. This study will also be conducted within the context of a larger project to more clearly understand the pest potential of *D. barberi* in Nebraska. Results from this project will contribute to the overall database needed to develop appropriate *D. barberi* management strategies.

Objectives

1) Evaluate the frequency of extended diapause within and among *D. barberi* populations from various regions of eastern Nebraska. This objective will determine the extent of extended diapause in eastern Nebraska populations and help factor out environmental vs. genetic contributions to recent increases in *D. barberi* densities observed in Nebraska.

2) Compare behavioral differences between a) *D. barberi* and *D. longicornis* and b) among *D. barberi* populations in Nebraska. Electroantennogram analysis will be used to measure the beetles responses to differing concentrations of the corn volatile Geranylacetone (brown corn silk volatile). If differences in response among species are found, then this may be able to be used as a tool to help document possible effects of introgression between species on the behavior of different *D. barberi* populations

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CHAPTER 2:

FREQUENCY OF EXTENDED DIAPAUSE OF *DIABROTICA BARBERI* SMITH AND LAWRENCE IN EASTERN NEBRASKA

Introduction

The northern corn rootworm, *Diabrotica barberi* Smith and Lawrence, is an important insect pest of corn, *Zea mays* L., in the western Corn Belt (Krysan 1986). *Diabrotica barberi* larvae feed on the roots of corn which can inhibit the plants ability to uptake water and nutrients from the soil, lead to plant instability (lodging), and cause yield loss (Woodson and Jackson 1996, Krysan et. al. 1983).

Diabrotica barberi is univoltine with initial egg hatch occurring in late May to early June in the U.S. Corn Belt with adult emergence often in July and August. Female *D. barberi* oviposit primarily in corn fields and place eggs in the soil (Boetel et al. 1992, Chiang 1973, Krysan 1999). *D. barberi* eggs enter obligatory diapause, which allows them to survive the winter (Krysan 1999).

Traditionally, crop rotation from a host to non-host crop (i.e., annual corn-soybean rotation) has been an effective way to manage *D. barberi* in corn as larvae could not survive on non-host roots. However, over the past 25 years, farmers in Minnesota, Iowa, and South Dakota have reported rootworm damage in first-year corn in annual rotation (Ostlie 1987). It has been shown that the most likely cause of this phenomenon is the presence of extended diapause in *D. barberi* populations (, Chiang 1965, Krysan et al. 1984, Krysan et al. 1986, Ostlie 1987, Levine et al. 1992). Extended diapause is a

process in which *D. barberi* remain in the egg stage for more than one winter (Ostlie 1987). Extended egg diapause in *D. barberi* was first reported by Chiang (1965) in Minnesota, but the frequency was only 0.3 percent of the population. Overtime, the frequency of extended diapause has increased to 40-50 percent in some areas (Krysan et al. 1984, Levine et al. 1992). Selection pressure on *D. barberi* created by crop rotation has been reported as a possible mechanism that has led to increases in the frequency of extended diapause in some populations (Ostlie 1987). Those that exhibit extended diapause would survive and therefore pass on the trait while those that do not would die when they hatch out near an unsuitable host (Ostlie 1987).

Over the last decade, northern corn rootworm densities and reports of rootworm injury in first-year corn have been increasing in eastern Nebraska (Hunt et al. 2007). The reason is unclear, but a series of warm winters which may have increased overwintering survival and possible increases in frequency of *D. barberi* extended diapause may be contributing factors. A 2006-2007 survey of *D. barberi* emergence patterns in first-year cornfields revealed that population densities are highly variable in eastern Nebraska but in general are much higher in the northern two thirds of the state than locations farther south (Hunt et al. 2007, Meinke et al. unpub.). The presence of *D. barberi* extended diapause has been confirmed and characterized in a population from Saunders County, Nebraska (Campbell 2009), but the frequency of extended diapause has not been evaluated in other *D. barberi* populations around the state. Therefore, as part of a larger study to more clearly understand the biology and pest potential of *D. barberi* in

Nebraska, a study was conducted from 2008-2010 to measure the frequency of extended diapause in populations of *D. barberi* from eastern Nebraska.

Materials and Methods

Gravid *D. barberi* females were collected from seven counties in eastern Nebraska: Sherman, Nance, Dixon, Saunders, Lancaster, Jefferson, Nemaha and one location in Brookings County, South Dakota, during August-September 2008 (Fig 1). Additional collections were made during August-September 2009 from the same seven Nebraska sites plus a site in Webster County Nebraska (Fig 1). Most collection sites were approximately 80-160 km (50-100 miles) apart.

Individual gravid females were placed in food/ovipositional boxes as described by Campbell and Meinke (2010). The polystyrene oviposition boxes were 5.9 cm long by 5.9 cm wide by 7.8 cm high, which included a lid 0.64 cm deep (ShowMan box, Althor Products, Wilton, CT). A food "shelf" made of a rectangular piece of plastic (4.5 cm long by 2.5 cm wide by 1.5 cm high) was attached to the lid with Velcro (Velcro USA, Manchester, NH). Boxes contained moist autoclaved soil (about 30% moisture by volume) that was pre-sifted through a 60-mesh sieve. The beetles were fed a diet consisting of field and sweet corn ear tissue that was changed every 3-5 days. The beetles were maintained in the ovipositional boxes until they died.

The eggs were separated from the soil by washing soil through a 60-mesh sieve. Eggs recovered on the sieve were placed on a milk filter (KenAG Animal Care Group,

product D110, Ashland, OH), and then counted under a stereomicroscope. After counting, eggs were placed back on autoclaved moist soil in Petri dishes and partially covered with a layer of 60-mesh soil.

Eggs from each female were held separately. Eggs for the 2008 collections were obtained from 13-22 females per site. Eggs for the 2009 collections were obtained from 13-15 females per site. Only boxes with a minimum of 50 eggs per female were used in the study. The overall mean number of eggs recovered per female was 176.76 ± 10.31 in 2008 and 153.09 ± 6.32 in 2009. Mean eggs obtained per female are presented in Tables 1 and 2, respectively, for each collection site and year.

To facilitate diapause development and termination, eggs were maintained at 22°C for 1 to 2 months after oviposition, 10°C for approximately 30 days, 5°C for about 6 months, and 22°C until eclosion of neonate larvae. Once egg hatch began, the Petri plates were checked daily to count and remove any neonate larvae present. Unhatched eggs were maintained at 22°C for 3-4 months and then subjected to the temperature profile listed above for a second year.

After the second-year egg hatch totals from the 2008 collections had been counted, the number of remaining eggs that appeared normal (milky white, whole eggs) were counted. The eggs were separated from the soil in the Petri dishes using the same method described above to recover eggs from ovipositional boxes.

Statistical Analysis

The proportion of eggs that hatched after one year (2008, 2009 collections analyzed separately), were statistically compared among sites with analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (SAS ver. 9.2, 2009). Mean maximum estimated egg viability after two years (hatched eggs + remaining normal unhatched eggs/initial egg sample size), and mean proportion remaining normal unhatched eggs after two years (remaining normal unhatched eggs/ initial egg sample size) from 2008 collections were also analyzed with ANOVA using PROC MIXED (SAS ver. 9.2, 2009) . The assumptions of normality and constant variance were evaluated using PROCUNIVARIATE and PROCGPLOT (SAS ver. 9.2, 2009). Because the assumptions were met, data were not transformed before analysis. Chi-Square analyses using PROCFREQ in SAS (SAS ver. 9.2, 2009) were performed to determine if there were significant differences within populations with regard to first-year percentage egg hatch. A significance level of $P<0.05$ was used in all analyses. For ANOVA, means were separated using Fisher's protected LSD test. Means and standard errors are reported as the LSMEANS obtained from analyses.

Results

The first-year average percentage hatch for the 2008 collection ranged from 4.5-38.6 % (Table 1). Sherman County, which was significantly different from all other populations collected that year, had the largest first-year average percentage hatch (38.6 ± 5.13). The second highest average percentage hatch came from Jefferson County, which

was also significantly different from all other counties. Egg hatch percentages recorded from Brookings, Nance, Saunders, Dixon, and Nemaha counties were not significantly different from each other. Lancaster County had the lowest average percentage hatch (4.5 ± 1.48) but was not significantly different from Nance, Saunders, Dixon, and Nemaha counties (Table 1). Second-year egg hatch occurred in every population from the 2008 collections which ranged from 5.3-15.9% of the original egg cohort sizes (Table 1).

The first-year percentage egg hatch for the 2009 collection ranged from 10.67-42.45% (Table 2). First-year average percentage egg hatch data from the 2009 collections followed a pattern that was similar to that observed from 2008 collections. The Sherman County site again had the highest mean percentage hatch (42.4 ± 3.44), but in this case, it was not significantly different than the Jefferson County site (38.6 ± 3.44). Mean percentage first-year egg hatch values from both Sherman and Jefferson counties were significantly greater than the mean percentage egg hatch from other collection sites. The mean from Webster County was significantly lower than means from Sherman and Jefferson counties but significantly greater than means from Dixon, Nemaha, and Lancaster Counties (Table 2). Lancaster, Nemaha, Dixon, Saunders, and Nance Counties exhibited the lowest mean percentage hatch values and were not significantly different from each other (Table 2).

The Lancaster, Nemaha, Saunders, and Nance 2008 collection sites had the highest mean proportion of remaining normal unhatched eggs after two years and were not significantly different from each other. The Sherman (0.04 ± 0.05) County site had

the lowest mean proportion remaining normal unhatched eggs which was significantly different than all other populations except Jefferson County (Table 1).

The estimated maximum egg viability, after completion of second-year hatch, was fairly consistent among the 2008 populations (Table 1). Most estimates of mean maximum egg viability ranged from 0.53 – 0.67. Only the Dixon population mean fell below this range and was significantly different from the other populations (Table 1). The Lancaster population had the greatest mean maximum egg viability (0.67 ± 0.04), but was not significantly different than means from Sherman, Saunders, and Nemaha populations (Table 1). The maximum estimated egg viabilities of the Brookings, Nance, Jefferson, Saunders, Nemaha, and Sherman populations were not significantly different from each other (Table 1).

All 2008 and 2009 populations had significant within population variation with regards to first-year percentage egg hatch (Tables 3 and 4). Figures 1 and 2 graphically present examples of the within population variation in first-year egg hatch from populations with relatively high (Sherman) and relatively low (Saunders, Nemaha) mean first-year percentage egg hatch.

Discussion

The data from this study collectively indicate that the extended diapause trait occurs in all *D. barberi* populations included in this study, and that significant differences in the frequency of the extended diapause trait occur among populations in eastern Nebraska. Several lines of evidence support this statement. The consistent significant

differences in percentage first-year egg-hatch among populations from 2008 and 2009 collections show that results were repeatable within sites (Tables 1 and 2). Mean estimated maximum egg viability was fairly consistent among populations (Table 1). The Sherman population which exhibited elevated mean percentage first-year egg hatch, was not significantly different in estimated maximum egg viability from six of the seven populations included in 2008 collections. This strongly supports the premise that first-year egg hatch variation among sites was due to differences in extended diapause expression and not variation in egg viability. Finally, the mean proportion of normal eggs remaining after the second-year hatch was completed strongly mirrored the first-year hatch data. A significantly lower proportion of normal eggs remained from the Sherman site than any other population (Table1). This study was terminated after two years but previous studies have shown that *D. barberi* is capable of remaining in diapause up to four years (Levine et. al. 1992, Campbell 2009). Populations such as Nemaha, Lancaster, and Saunders appear to have many viable eggs that could still hatch in year three while potential egg hatch from the Sherman site is nearly completed.

The data support the hypothesis that the high frequency extended diapause trait may have moved down from South Dakota/Minnesota into Nebraska over time. In general, the highest frequency of extended diapause appears to occur in a north-south transect from Brookings Co. SD to Nemaha Co. NE that parallels the Missouri River. *D. barberi* populations collected farther west from the north-south transect exhibited lower frequencies of extended diapause. It is unclear why the current geographic patterns of extended diapause exist but several factors may have contributed to the trends observed.

If the high frequency extended diapause trait followed a diffusion pattern from the north, it would be easier to follow a natural corridor along a river system than to move west against prevailing summer winds. Also, central Nebraska is at the western edge of the geographical range of the species (Krysan and Smith 1987) where populations are spotty and densities are often low which could isolate populations from the main North-South transect. Growers in central Nebraska historically have grown continuous corn in contrast to growers in very eastern Nebraska, which have commonly followed a corn-soybean annual rotation. The latter scenario would favor selection for extended diapause (Krysan et al. 1986).

This study provides another example of genetic variation that can occur both within and among *D. barberi* populations. Previous research has shown that *D. barberi* can exhibit relatively high levels of genetic diversity and variation of traits across the United States. Several studies have documented that significant genetic differences can occur among *D. barberi* populations that are in close geographic proximity such as aldrin susceptibility (Ball and Weekman 1963, Krysan and Sutter 1986), amplified fragment lenth polymorphism (AFLP) variation (Campbell et al. 'in press'), and polymorphic allozyme loci (Krafsur et. al. 1993). It has been suggested that small-scale variation could be due in part to limited flight activity of *D. barberi* which may lead to partially isolated breeding populations (McDonald et. al. 1985, Krafsur 1993). *D. barberi* typically moves outside of cornfields in order to find new sources of food (Cinereski and Chiang 1968, Naranjo and Sawyer 1988). However, tethered flight studies have shown

that *D. barberi* typically only takes short trivial flights and does not undertake long range migratory flights (Naranjo 1990).

Results from this study contribute to the database needed to develop appropriate *D. barberi* management recommendations for different areas of eastern Nebraska. Because the extended diapause trait appears to occur throughout eastern Nebraska, it is important to note that growers utilizing crop rotation in Nebraska to manage rootworms should expect to see some *D. barberi* present in first-year corn. A better understanding of the extent of extended diapause in an area can also help to determine the effectiveness of crop rotation as a rootworm management tactic. In areas with a high frequency of extended diapause expression crop rotation may not always be enough to keep *D. barberi* populations from causing economic damage in first-year corn when *D. barberi* densities are high. Growers in areas west of the north-south transect should be able to control *D. barberi* populations using crop rotation because of a lower presence of extended diapause and relatively low *D. barberi* densities.

From a resistance management perspective, the occurrence of extended diapause in *D. barberi* populations may be a positive attribute. If conventional insecticides or plant incorporated protectants (i.e., transgenic events) are used as part of rootworm management programs, *D. barberi* populations emerging two, three, or four years later would provide a natural refuge source. *D. barberi* adults would be emerging in the same geographic area over time with little previous exposure to the pesticides or specific transgenic events being used there. These beetles could potentially mate with individuals that are offspring of more highly selected individuals effectively lowering the potential of

resistance evolution in an area. This benefit would be in addition to any structured refuge that may be in place.

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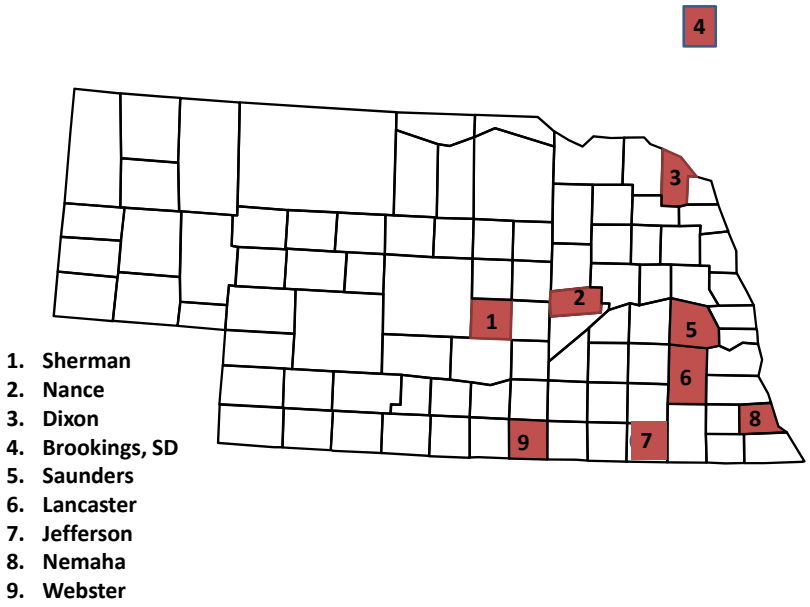

Figure 1. Nebraska counties where *Diabrotica barberi* females were collected.

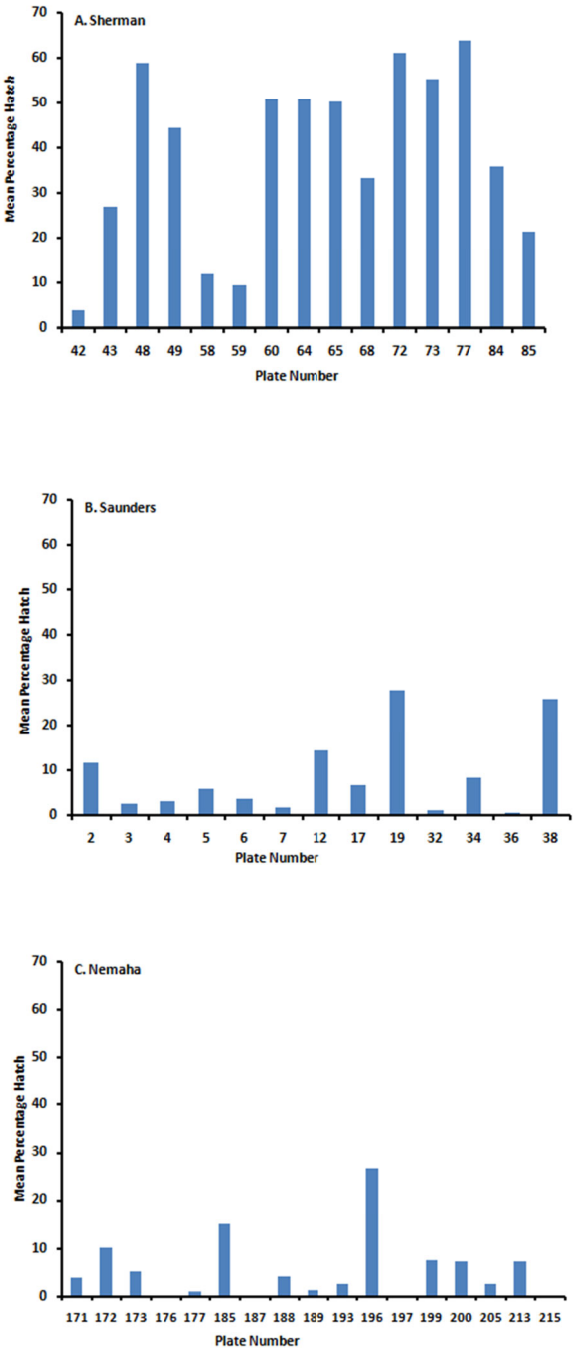

Figure 2. 2008 northern corn rootworm collection: percentage first-year egg hatch from egg cohorts obtained from individual females; **A.** Sherman site **B.** Saunders site, and **C.** Nemaha site.

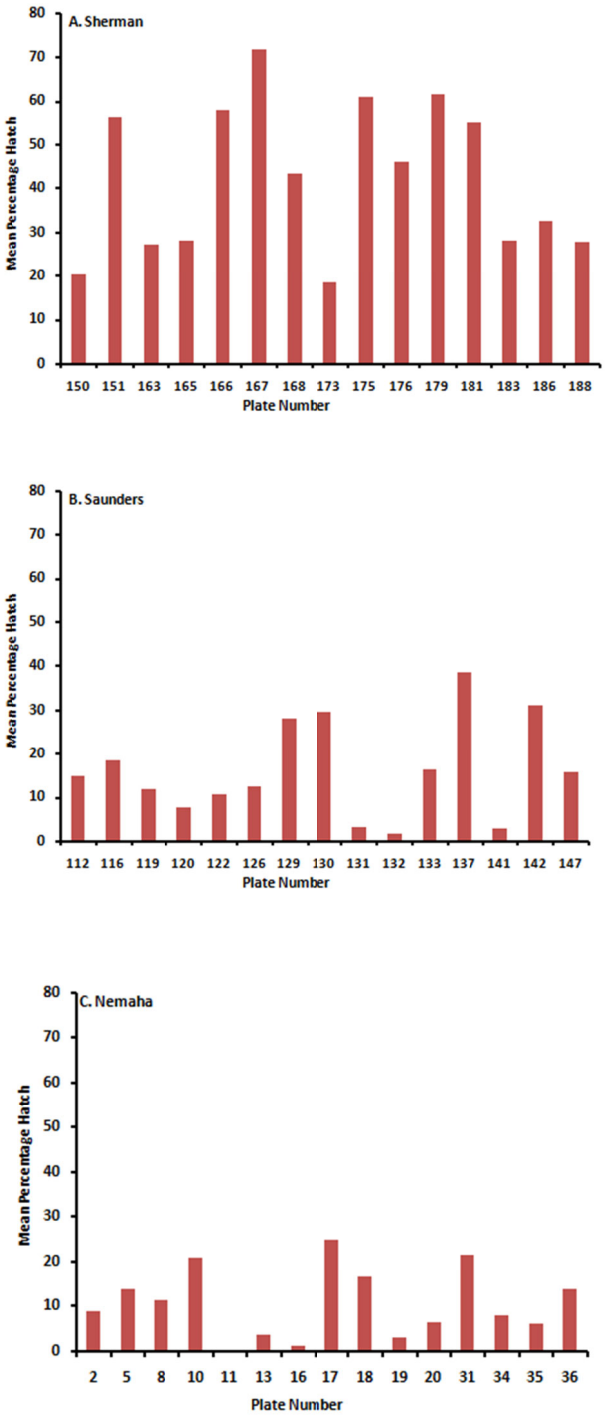

Figure 3. 2009 northern corn rootworm collection: percentage first-year egg hatch from egg cohorts obtained from individual females; **A.** Sherman site **B.** Saunders site, and **C.** Nemaha site.

Table 1. 2008 Collections. Mean northern corn rootworm egg cohort size, percentage egg hatch, and estimated maximum egg viability per site.

Collection Site	N	Mean Eggs Per (♀) ± SE	Mean First Year Percentage Egg Hatch ± SE [a]		Mean Second Year Percentage Egg Hatch ± SE [b]		Post Year 2 Hatch Mean Estimated Egg Max Viability ± SE [c]		Post Year 2 Hatch Mean Proportion Normal Eggs Remaining ± SE [d]	
Lancaster	22	103.55 ± 21.92	4.46 ± 2.32	A	5.33 ± 1.43	A	0.67 ± 0.04	C	0.57 ± 0.05	D
Nemaha	17	113.94 ± 24.94	5.56 ± 2.63	AB	7.89 ± 1.63	AB	0.62 ± 0.05	BC	0.49 ± 0.05	D
Dixon	14	156.00 ± 27.48	6.29 ± 2.90	AB	6.31 ± 1.79	A	0.35 ± 0.05	A	0.22 ± 0.05	BC
Saunders	13	262.08 ± 28.52	8.69 ± 3.01	AB	6.84 ± 1.86	A	0.64 ± 0.05	BC	0.49 ± 0.06	D
Nance	15	228.73 ± 26.55	10.37 ± 2.80	AB	8.63 ± 1.73	ABC	0.53 ± 0.05	B	0.34 ± 0.05	CD
Brookings	16	261.75 ± 25.70	12.69 ± 2.72	B	15.86 ± 1.68	D	0.54 ± 0.05	B	0.26 ± 0.05	BC
Jefferson	15	170.33 ± 26.55	23.09 ± 2.80	C	12.43 ± 1.73	BC	0.54 ± 0.05	B	0.18 ± 0.05	AB
Sherman	15	164.53 ± 26.55	38.56 ± 2.80	D	13.02 ± 1.73	CD	0.56 ± 0.05	BC	0.04 ± 0.05	A
P			<0.0001		<0.0001		0.0003		<0.0001	
F			17.98		5.19		4.23		13.12	
df			7, 119		7, 119		7, 119		7, 119	

Means presented are least-squares means (LSMEANS); Within columns, means followed by the same upper-case letter are not significantly different (P > 0.05).

N represents the number of females per site that laid > 50 eggs; egg cohorts were then used in the diapause study.

a. Mean first-year percentage egg hatch = (Site total hatch/Site total eggs collected) x 100.

b. Mean second-year percentage egg hatch = (Site total hatch/Site total eggs collected) x 100.

c. Post year 2 hatch mean estimated egg maximum viability = (hatched eggs + remaining normal unhatched eggs/ initial egg sample size); normal eggs = milky white, whole eggs.

d. Post year 2 hatch mean proportion normal eggs remaining = (remaining normal unhatched eggs/ initial egg sample size).

Table 2. 2009 collections. Mean northern corn rootworm egg cohort size and percentage egg hatch.

Collection Site	N	Mean Eggs Per (♀) ± SE	Mean First Year Percentage Hatch ± SE[a]	
Lancaster	13	101.08 ± 16.75	12.18 ± 3.69	A
Nemaha	15	127.67 ± 15.59	10.67 ± 3.44	A
Dixon	15	149.27 ± 15.59	11.61 ± 3.44	A
Saunders	15	114.07 ± 15.59	16.25 ± 3.44	AB
Nance	15	211.80 ± 15.59	16.65 ± 3.44	AB
Webster	15	172.87 ± 15.59	25.24 ± 3.44	B
Jefferson	15	148.07 ± 15.59	38.60 ± 3.44	C
Sherman	15	193.00 ± 15.59	42.45 ± 3.44	C

Means presented are least-squares means (LSMEANS).
Within first-year hatch column, means followed by the same uppercase letter are not significantly different.
N represents the number of females per site that laid > 50 eggs; egg cohorts were then used in the diapause study.
a. Mean first-year percentage egg hatch = (Site total hatch/Site total eggs collected) x 100.

Table 3. Results of Chi-Square analysis of within population variation of first-year egg hatch data, 2008 collections.

Collection Site	N	Df	Chi-Square Value	Chi-Square Probability
Lancaster	22	21	230.81	<0.0001
Nemaha	17	16	129.54	<0.0001
Dixon	14	13	139.33	<0.0001
Saunders	13	12	321.11	<0.0001
Nance	15	14	199.66	<0.0001
Brookings	16	15	336.43	<0.0001
Jefferson	15	14	260.91	<0.0001
Sherman	15	14	388.71	<0.0001

N represents the number of females per site that laid > 50 eggs; egg cohorts were then used in the diapause study.

Table 4. Results of Chi-Square analysis of within population variation of first-year egg hatch data, 2009 collections.

Collection Site	N	Df	Chi-Square Value	Chi-Square Probability
Lancaster	13	13	117.36	<0.0001
Nemaha	15	15	106.34	<0.0001
Dixon	15	15	361.63	<0.0001
Saunders	15	15	162.47	<0.0001
Nance	15	15	376.93	<0.0001
Webster	15	15	291.63	<0.0001
Jefferson	15	15	147.17	<0.0001
Sherman	15	15	341.13	<0.0001

N represents the number of females per site that laid > 50 eggs; egg cohorts were then used in the diapause study.

Appendix 1

COMPARATIVE ELECTROANTENNOGRAM ANALYSIS OF *DIABROTICA BARBERI* SMITH AND LAWRENCE, AND *DIABROTICA LONGICORNIS* (SAY) RESPONSE TO THE CORN VOLATILE GERANYLACETONE

Introduction

In 1820 Thomas Say collected a beetle off of a wild cucurbit and named it *Diabrotica longicornis* (Say 1824). In the 1870s, a rootworm species was documented feeding on continuous corn, *Zea mays* L., in Illinois and Missouri, which morphologically was very similar to *Diabrotica longicornis* (Say) (Krysan et al. 1983). Because of the similarity, the rootworm found on corn was also called *D. longicornis* (Webster 1913, Krysan et al. 1983). Smith and Lawrence (1967) subsequently described the subspecies, *D. longicornis longicornis* and *D. longicornis barberi* which separated the non-pest from the pest species, respectively. *Diabrotica barberi* Smith and Lawrence, and *Diabrotica longicornis* (Say) were elevated to species rank by Krysan et al. (1983) based on differences in behavior (e.g., pheromone response, unidirectional hybridization, and habitat choice), range, and color between the two taxa. Since that time, genetic evidence has been reported that documents the occurrence of gene flow among taxa (introgression) in the area of sympatry which includes parts of eastern Kansas and eastern Nebraska (Campbell 2009, Campbell et al. "in press").

Habitat preferences and potential host utilization by each species are not clearly understood (Campbell et al. "in press") but may be important factors that could separate

the species. *Diabrotica barberi* is closely associated with corn (Branson and Ortman 1971, Branson and Ortman 1967, Naranjo 1994), causing damage through larval feeding on the roots of the plant (Chiang 1973). *Diabrotica longicornis* does not utilize corn as a larval food source in the field and adults have only rarely been observed to feed on corn (Branson and Krysan 1981). However, it is possible to rear *D. longicornis* on corn in the laboratory (Golden and Meinke 1991), which raises the question as to why *D. longicornis* would not utilize such a widespread food source in the field. One factor involved may be variation in relative attractiveness of each species to the volatiles of corn. Geranylacetone, (+)-a-terpineol, and linalool are corn volatiles that have been found to be attractive to *D. barberi* beetles in the field. (Hammack 1997, Hammack 1996). Geranylacetone, which is synthesized from the brown silks of corn, was shown to be highly attractive to both male and female *D. barberi* (Hammack 1997, Hammack 1996). No studies have been conducted to quantify the response of *D. longicornis* to any volatiles of corn.

To begin to explore the potential differences in response between species to a known corn volatile, a preliminary experiment was conducted to measure the antennal response of *D. longicornis* and *D. barberi* to varying concentrations of the corn volatile geranylacetone using electroantennogram (EAG) analysis. The objectives of the experiment were to determine if 1) a dose of geranylacetone could be identified that would elicit a different response in either males or females between species; and 2) determine if the EAG technique shows promise as a tool to investigate possible introgression between the two taxa in future studies.

Materials and Methods

Adult *D. barberi* beetles were collected from four counties during August-September 2010 (eastern Nebraska: Dixon, Saunders, Nemaha; South Dakota: Brookings, Fig 1 in chapter 2). Additional *D. barberi* were reared out from adult collections made during 2009 from Jefferson and Webster counties. A single *D. longicornis* population was collected off of buffalo gourd flowers, *Cucurbita foetidissima* HBK in Dundy County Nebraska in 2010. EAG analysis was then conducted on six *D. barberi* populations and one *D. longicornis* population. Four treatments were evaluated to compare relative response between populations. Treatments included: a pure air control, a 1µl/µg Geranylacetone in 99µl/µg hexane mixture (1/100), a 1µl/µg Geranylacetone in 999µl/µg hexane mixture (1/1000), and a pure hexane control. The available number of males and females from each population were subjected to each treatment (Tables 3 and 4).

Rearing

The procedure used to maintain adult *D. barberi* and obtain viable eggs from females was previously described in chapter 2. The larvae obtained from eggs were reared to adults using the same method as described by Campbell and Meinke (2010). Larvae were reared individually in 1 oz plastic cups with lids (SYSCO, Houston, TX, and Sweetheart (Solo), Highland Park, IL) and provided with corn seedlings as a food source. Over the course of its development, each larva received three fresh corn seedlings; as the corn germinated it provided new roots for larval feeding and development. Pioneer

Brand 31G66 (Johnston, IA) corn, treated with fungicides Fludioxonil and Mefenoxam, was used for rearing. Two-thirds of each cup was filled with peat moss and planted with corn seedlings. Peat moss was mixed with distilled water to 30% moisture by volume, prior to placement in cups and planting. Larvae were reared at room temperature, approximately 22 °C. As adults emerged, gender was determined using the method of White (1977). Individual beetles were kept alive in empty 20.57 cm^3 cups and fed a steady diet of fresh lettuce (*Lactuca sativa* L.).

Volatile Preparation

Synthetic Geranylacetone (Sigma-Aldrich, Saint Louis, MO) was diluted with hexane (Sigma-Aldrich Saint Louis, MO) into two concentrations: 1µl/µg of volatile to 99µl/µg of hexane and 1µl/µg of volatile to 999µl/µg of hexane, respectively. Ten µl of each treatment was placed on a separate small piece of filter paper (≈1 x 5cm). The solvent was allowed to completely evaporate usually taking about 10 minutes. Afterwards, each slip was placed in an individual Pasteur pipette. One additional pipette contained only an untreated slip of filter paper to serve as a pure air control. Fresh samples were made each day for use in electroantennogram assays.

Antennal Preparation

Antennal preparation was done using a modified version of the methods presented in the Syntech 2004 electroantennogram manual and Hibbard et. al. (2002). Using a pair of fine tipped scissors, the head was removed and the distal tip of the antenna was removed. Glass capillary filaments (1.5mm x .86mm, 10.16cm) that had been modified

to come to a point at one end were filled with conductive gel (Spectra 360, Parker Laboratories, Inc) using a syringe being sure not to leave any air bubbles. The severed beetle head was placed on the gel extruding from the pointed end of one of the filaments. The filament was then slid onto the reference electrode. The other filament was slid onto the recording electrode. At this point the electrodes were adjusted until the excess gel from the recording electrodes filament came in contact with the cut tip of the beetle's antenna.

Stimulus Delivery

Once everything was in place a glass tube was pointed at the antenna. The tube was connected to an air pump which blew constant purified air (≈25cm/sec) over the antenna. The volatile and control samples were individually mixed into the air flow by connecting another air tube to the Pasteur pipette and placing the other end into a small aperture in the main tube pointed at the antennae. The air flow to the pipette was controlled by a switch that allowed only a quick burst of air (≈0.3 to 0.5 sec long). A rest period of thirty seconds was given between each stimulus. The reaction of each stimulus was recorded by the electroantennogram and measured in micro volts using the program AUTOSPIKE (Syntech 2004). Spike measurements were made by calculating the distance between the base of the spike to the highest point of the spike.

Statistical Analysis

All EAG data were analyzed in SAS version 9.2 (2009). An initial exploratory analysis of variance (ANOVA) of the main effects site, sex, and treatment, as well as possible interactions between main effects, were analyzed using the PROC MIXED procedure. Because of the results of the exploratory ANOVA, a simple ANOVA (PROC MIXED Procedure) was also used to analyze only the main effect site for each sex within the 1/100 geranylacetone/hexane treatment. The PROC GLIMMIX procedure was used to make simple effect comparisons for main effects sex and treatment means. A significance level of $P<0.05$ was used in all analyses. For analysis of variance, means were separated using Fisher's protected LSD test. Means and standard errors were obtained from the LSMEANS statement in the PROC MIXED procedure (Littell et al. 2006).

Results

Results of the exploratory overall ANOVA indicate that there were significant differences within sites, sex, and treatments (Table 1). There were also significant site by treatment, sex by treatment, and site by sex by treatment interactions, but not a significant site by sex interaction (Table 1). Across sites and sex, the magnitude of EAG response was significantly greater in the 1/100 geranylacetone treatment than the other treatments tested (Table 2). Males showed a significantly stronger EAG response than females across all sites and treatments (mean EAG responses: male: 653.4 ±62.3μV, female: 419.9± 42.1μV). Results of the ANOVA analysis of EAG response data from within the

1/100 geranylacetone treatment indicate that males from Dundy County exhibited significantly greater reaction spikes to geranylacetone than all other populations except Webster (Table 3). A similar analysis of female data indicated that there were not significant differences in response to geranylacetone among populations (Table 4).

Discussion

The data presented in this experiment shows that male *D. longicornis* collected from Dundy County Nebraska had a stronger response to the 1/100 geranylacetone treatment than any the *D. barberi* populations tested except Webster (Table 3). The responses from the *D. barberi* collected from Webster County tracked much closer to the responses of the *D. longicornis* population from Dundy County than those of the other *D. barberi* populations, although Webster was not significantly different than D. barberi populations. The reasons for this are not clear; however, Webster County is geographically the closest population to the Dundy County site and Webster County falls within the overlap zone of the two species where introgression may occur (Campbell et al. "in press"). Preliminary data from this experiment suggest that male EAG response data may be useful as a possible method for differentiating *D. barberi* from *D. longicornis* and warrants further study to determine if the tool could be used to study *D. longicornis*/*D. barberi* introgression in the area of sympatry. The method however, is still too inconsistent (data was highly variable) and would need to be optimized and confirmed as a diagnostic tool before this would be possible. To further test this idea several things should be done to improve the study. Using larger sample sizes with a 50/50 male female ratio and standardized diet and age of beetle cohorts may reduce

variability in results. Additional range finding for the volatile concentrations would also help to optimize effective dose and possibly identify female diagnostic concentrations between taxa as well. Other known corn volatiles could also be screened.

While the EAG analysis revealed possible differences in antennal response between *D. longicornis* males and *D. barberi* males, it does not provide evidence as to how the beetles would react to geranylacetone in the field. In order to determine how the beetles behaviorally would react to the volatile, additional experiments would have to be performed. Examples include lab studies such as a Y-tube bioassay, in which the beetles are given a choice between the volatile or a control. Field studies could also be performed, such as a field capture study utilizing the volatile as bait in different geographic areas.

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Table 1. Electroantennogram data analysis; results of ANOVA for the main effects site, sex, and treatment plus interactions.

Effect	NumDf	Den Df	F Value	Pr> F
Site	6	92	3.42	0.0043
Sex	1	92	9.65	0.0025
Site x Sex	6	92	1.33	0.25
Treatment	3	276	88.21	<0.0001
Site x Treatment	18	276	5.87	<0.0001
Sex x Treatment	3	276	19.61	<0.0001
Site x Sex x Treatment	18	276	3.52	<0.0001

Table 2. Magnitude of EAG response per site (LSMEAN ± SE) across sites and gender.

Treatment	Df	Mean ± SE	
Air	211.3	353.26 ± 47.62	A
Hexane	211.3	383.73 ± 47.62	A
1/100	211.3	1011.64 ± 47.62	AB
1/1000	211.3	398 ± 47.62	A

1/100 represents the 1µl/µg Geranylacetone, 99µl/µg Hexane treatment.
1/1000 represents the 1µl/µg Geranylacetone, 999µl/µg Hexane treatment.

Table 3. Magnitude of EAG response per site (LSMEAN ± SE) by males to 1/100 geranylacetone treatment.

Site	N	Mean ± SE	
Brookings	1	740 ± 0	A
Dixon	5	904 ± 156.77	A
Jefferson	5	988 ± 222.76	A
Saunders	5	1114 ± 311.99	A
Nemaha	8	1320 ± 197.70	A
Webster	2	1820 ± 940	AB
Dundy	16	2575 ± 264.11	B

1/100 represents the 1µl/µg Geranylacetone, 99µl/µg Hexane treatment.
Beetles tested from Dundy County were *D. longicornis.*
Beetles tested from Brookings, Dixon, Jefferson, Saunders, Nemaha, and Webster counties were *D. barberi.* Male EAG response was significantly different among sites ($F = 5.26$, $df = 6, 35$, $P = 0.0006$).

Table 4. Magnitude of EAG response per site (LSMEAN ± SE) by females to 1/100 geranylacetone treatment.

Site	N	Mean ± SE	
Brookings	14	491.43 ± 44.95	A
Dixon	5	648 ± 56.43	A
Jefferson	12	545 ± 40.16	A
Saunders	16	647.5 ± 110.63	A
Nemaha	3	706.66 ± 152.46	A
Webster	6	793.33 ± 152.72	A
Dundy	8	870 ± 122.24	A

1/100 represents the 1µl/µg Geranylacetone, 99µl/µg Hexane treatment.
Beetles tested from Dundy County were *D. longicornis.*
Beetles tested from Brookings, Dixon, Jefferson, Saunders, Nemaha, and Webster counties were *D. barberi.*
Female EAG response was not significantly different among sites ($F=1.80$, $df = 6, 57$, $P=0.1155$).

Appendix 2. SAS code of ANOVA for mean percentage hatch, estimated proportion remaining live eggs, and estimated max egg viability.

```
data egg2008;
input site $ prop;
datalines;
;
proc mixed;
class site;
model prop=site;
lsmeans site/diff;
proc means n mean stderr;
by site;
var prop;
run;
```

Appendix 3. SAS code of ANOVA for within site mean percentage hatch.

```
data mead;
input female hatch$ count;
datalines;
;
proc freq data=mead;
tables female * hatch / chisq;
weight count;
run;
```

Appendix 4. SAS code of ANOVA for the main effects of the EAG procedure.

```
 data eag;
input site $ beetle sex $ trt $ y;
datalines;
;
proc mixed;
class site beetle sex trt;
model y=site | sex | trt/ddfm=kr;
random beetle (site);
lsmeans site*sex*trt/slice=sex*trt;
run;
proc glimmix;
class site beetle sex trt;
model y=site | sex | trt/ddfm=kr;
random beetle (site);
lsmeans site*sex*trt/slicediff=sex*trt;
lsmenas site sex trt/diff;
run;
```

Appendix 5. SAS code of ANOVA for just the (1/100) treatment of the EAG procedure.

```
data eag;
input site $ beetle sex $ trt $ y;
datalines;
;
proc mixed;
class site beetle;
model y=site/ddfm=kr;
random beetle (site);
lsmeans site;
run;
proc glimmix;
class site beetle;
model y=site;
random beetle (site);
lsmeans site;
lsmenas site/diff;
proc means n mean stderr;
by site;
var y;
run;
```