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# Fall Armyworm (Lepidoptera: Noctuidae) and Southwestern Corn Borer (Lepidoptera: Crambidae) Leaf Feeding Damage and Its Effect on Larval Growth on Diets Prepared from Lyophilized Corn Leaves[1]

W. Paul Williams[2,3] and Paul M. Buckley[3]

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**ABSTRACT** Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and Southwestern corn borer, *Diatraea grandiosella* Dyar, are economically important pests of maize (*Zea mays* L.) in the southeastern United States. These insects attack plants in both the vegetative and reproductive stages of growth. Plant resistance is widely considered a desirable means for reducing losses to both insects, and corn germplasm lines with resistance to leaf feeding damage have been developed and released. Fall armyworm and southwestern corn borer larvae feeding on resistant genotypes grow more slowly than those feeding on susceptible genotypes. The objectives of the investigation were to evaluate 20 single cross maize hybrids for leaf feeding damage by southwestern corn borer and fall armyworm, to compare larval growth on laboratory diets prepared from lyophilized leaf tissue of single cross hybrids with different levels of resistance to leaf feeding, and to determine whether larval growth differed between diets prepared from leaf tissue collected from plants previously infested with southwestern corn borer or fall armyworm and non-infested plants. Both fall armyworm and southwestern corn borer larvae weighed significantly less when fed on laboratory diets prepared from lyophilized leaf tissue of resistant single cross hybrids than susceptible hybrids. When tissue from either resistant or susceptible plants that had been infested with either insect in the field was used in bioassays, larval weights were further reduced. It appears that both constitutive and induced defensive mechanisms may be operating.

**KEY WORDS** *Diatraea grandiosella*, *Spodoptera frugiperda*, *Zea mays*, Lepidoptera, Crambidae, Noctuidae, host plant resistance

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and Southwestern corn borer, *Diatraea grandiosella* Dyar, are economically important pests of maize (*Zea mays* L.) in the southeastern United States. These insects attack plants in both the vegetative and reproductive stages of growth. Plant resistance is widely considered a desirable means for reducing losses to both insects. Breeding programs have been established at several locations to identify and develop maize germplasm with resistance to these and other Lepidoptera (Smith et al. 1989,

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Widstrom 1989, Williams & Davis 1997). Some of the germplasm lines developed as sources of resistance to southwestern corn borer and fall armyworm also exhibited resistance to sugarcane borer, *Diatraea saccharalis* (Fabricius), corn earworm, *Helicoverpa zea* (Boddie), and European corn borer, *Ostrinia nubilalis* (Hübner) (Davis et al. 1988).

In field tests resistant genotypes not only sustained less damage from larval feeding, but larval growth and survival were also reduced on plants of resistant genotypes (Williams et al. 1989). Differences in growth of southwestern corn borer, fall armyworm, and corn earworm larvae on resistant and susceptible maize genotypes were exhibited in laboratory bioassays using both callus tissue and diets prepared from lyophilized leaf tissue (Williams et al. 1987, 1995, Buckley et al. 1991, Williams & Buckley 1992). Differences in larval growth on susceptible and resistant genotypes in both field trials and laboratory bioassays have been the basis for experiments conducted to determine the factors that characterize resistant genotypes and distinguish them from susceptible genotypes.

A comparison of anatomical characteristics of susceptible and resistant inbred lines revealed that the most significant difference was the thicker cuticle and epidermal cell wall of the upper and lower leaf surfaces of resistant genotypes (Davis et al. 1995). Inner whorl tissue of resistant genotypes also tended to be tougher than that of susceptible genotypes. Hedin et al. (1984) reported that crude fiber and hemicellulose were consistently higher in whorl leaves of resistant genotypes. A subsequent investigation revealed that hemicellulose was not only more abundant in resistant plants, but also structurally different from that in susceptible plants (Hedin et al. 1996). Fall armyworm larvae reared on laboratory diets containing hemicellulose from susceptible genotypes weighed significantly more than those reared on diets containing hemicellulose from resistant genotypes.

Plant defenses may change with growth and maturation of the plants. Davis et al. (1999) and Chang et al. (2000) reported that fall armyworm larvae fed on excised leaf sections of the yellow portion of whorl leaves grew equally well on resistant and susceptible genotypes. When fed in the yellow-green or green portions of the leaves, larvae that fed on resistant genotypes weighed significantly less that those that fed on susceptible genotypes. In laboratory bioassays using lyophilized whorl tissue harvested from resistant and susceptible genotypes weekly from 28 to 56 d after planting, both fall armyworm and southwestern corn borer larval growth declined as plants matured (Williams et al. 1998). Differences between growth for larvae reared on resistant and susceptible genotypes also decreased at the later harvest dates.

An analysis of protein patterns of the callus of resistant and susceptible maize inbreds by 2-dimensional gel electrophoresis indicated the presence of a 33-kDa cysteine protease in the resistant inbred lines Mp704 (Williams & Davis 1984) and Mp708 (Williams et al. 1990) that was not present in the susceptible inbred lines Ab24E and Tx601 (Jiang et al. 1995). In a laboratory bioassay, there was a significant negative correlation between the concentration of the 33-kDa cysteine protease and the weight of fall armyworm larvae feeding on callus of $F_2$ progeny of the cross Mp704 $\times$ Tx601. After its discovery in callus tissue, Pechan et al. (2000) demonstrated that the 33-kDa cysteine protease accumulated in whorls of resistant plants in response to larval feeding. The abundance of the protease

increased dramatically 1 h after infestation and continued to accumulate for as long as 7 d.

In an attempt to determine how the cysteine protease affected the development of fall armyworm larvae, Pechan et al. (2002) used scanning electron microscopy to examine the midgut of larvae that had fed on resistant and susceptible plants. Their results indicated that the peritrophic membrane, which surrounds the food bolus and assists in digestion, was severely damaged when larvae fed on resistant plants or callus tissue expressing the 33-kDa cysteine protease. Conversely, when the peritrophic membranes of southwestern corn borer larvae that had fed on resistant and susceptible maize plants were examined, differences in degree of membrane damage associated with resistant and susceptible genotypes were not detected (Daves et al. 2007).

The overall objective of this research was to determine whether maize plants that have been fed on by fall armyworm or southwestern corn borer larvae became less susceptible and, if so, did the response differ among genotypes. The specific objectives of the investigation were (1) to evaluate 20 single cross maize hybrids for leaf feeding damage by southwestern corn and fall armyworm, (2) to compare southwestern corn borer and fall armyworm larval growth on laboratory diets prepared from lyophilized leaf tissue of single cross hybrids with different levels of resistance to leaf feeding, and (3) to determine whether larval growth differed between diets prepared from leaf tissue collected from plants previously infested with southwestern corn borer or fall armyworm and non-infested plants.

## Materials and Methods

Twenty single cross maize hybrids were grown at the R. R. Foil Plant Science Research Center at Mississippi State University, Mississippi State, Mississippi, in 2006 and 2007. The hybrids were selected because they exhibit a range in susceptibility to leaf feeding by fall armyworm and southwestern corn borer. Several of the parental inbred lines were developed and released as sources of resistance to leaf feeding: Mp496 (Scott & Davis 1981), Mp703 (Williams et al. 1980), Mp704 (Williams & Davis 1982), Mp707 (Williams & Davis 1984), Mp708 (Williams et al. 1990), Mp713 & Mp714 (Williams & Davis 2000), and Mp716 (Williams & Davis 2002).

**Evaluation of fall armyworm and southwestern corn borer leaf feeding damage.** Leaf feeding damage by fall armyworm and southwestern corn borer was evaluated in separate experiments planted on 13 April 2006 and 20 April 2007. Hybrids were planted in single-row plots with 20 plants per row. Rows were approximately 4 m long and 0.97 m apart. Within each experiment, hybrids were arranged in a randomized complete block design with three replications. On 18 May 2006 and 24 May 2007, the experimental plots were infested with either 30 fall armyworm or 30 southwestern corn borer neonates per plant. The larvae were obtained from cultures maintained by the USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State, MS. Larvae were mixed with corn cob grits and dispensed into the plant whorl using a mechanical applicator (Wiseman et al. 1980). In 2006, leaf feeding damage was visually rated 14 d after infestation, and in 2007, 12 d after infestation using the rating scales described by Williams et al. 1989. Fall armyworm damage was rated on a scale of 0, no damage, to 9, many leaves destroyed. Southwestern corn borer damage was

rated on a scale of 0, no damage, to 9, long lesions on most leaves. Rating scales for the two insects differ to accommodate variations in their patterns of damage.

**Laboratory assays of larval growth.** After leaf feeding damage was rated in 2007, whorls of seven of the single cross hybrids were harvested from the experiments that had been infested with fall armyworm or southwestern corn borer and from a similarly designed experiment that was not infested. The seven hybrids were selected to represent a broad range of susceptibility to leaf feeding. The whorls were removed from the plants and trimmed to approximately 15 cm in length so that the yellow-green portion of the leaves was retained. The yellow-green portion of the leaf is the primary larval feeding site. The tissue was placed in plastic freezer bags, packed in ice, and transported to the laboratory. The tissue was stored at −18°C until it was lyophilized, then ground to a fine powder using a laboratory mill with a 1-mm mesh screen.

Fall armyworm and southwestern corn borer larval growth was determined in laboratory bioassays conducted following the procedures described by Buckley et al. (1991). Diets were prepared by combining 250 mL distilled water, 2.4 g agar, 12.5 mg gentamicin sulfate (an antibiotic), 132 mg sorbic acid (an antifungal agent), and 528 mg ascorbic acid. The mixture was heated to 82°C while stirring. As stirring continued, 10 g of lyophilized leaf tissue was added to the mixture that was then dispensed in 10-mL aliquots into 30-mL plastic diet cups.

On 13 March 2008, 20 cups of diet were prepared from tissue of each of the selected single cross hybrids harvested from each of the experiments (fall armyworm infested, southwestern corn borer infested, and non-infested). On the following day, each cup was infested with one southwestern corn borer neonate and covered with a paperboard lid. Cups were arranged in a randomized complete block design with five replications of four cups each, and placed in an environmental chamber maintained at 28°C and a photoperiod of 12:12 (L:D). The larvae were weighed after 14 d. On 21 March, a similar experiment was initiated for evaluating fall armyworm growth. Because of their faster rate of growth, fall armyworm larvae were weighed after 10 d.

**Statistical analysis.** Data for leaf feeding damage ratings and larval weights in the laboratory bioassays were analyzed by analysis of variance (ANOVA) (SAS Institute 2003). Means were compared by Fisher's protected least significance difference (LSD) test (Steel & Torrie 1980).

## Results and Discussion

**Fall armyworm leaf feeding damage evaluations.** Damage ratings were higher in 2006 than 2007 (Table 1). This is probably due in part to the fact that damage was rated 14 d after infestation in 2006 and 12 d after infestation in 2007. Damage was rated earlier in 2007 so that the whorls could be collected for the bioassays while larvae were actively feeding. The overall mean damage rating was 8.0 in 2006 and 5.5 in 2007. In the analysis of data combined over years, hybrids ($F$ = 36.02; df = 19, 76; $P < 0.0001$), years ($F$ = 45.89; df = 1, 4; $P < 0.005$), and hybrids × years ($F$ = 13.42, df = 19, 76; $P < 0.0001$) were significant sources of variation. The susceptible hybrid SC229 × Tx601 sustained the heaviest damage with a rating of 9 each year. This indicates that many leaves were destroyed. Other hybrids that rated 9 in 2006 were also heavily damaged in

**Table 1. Fall armyworm leaf feeding damage sustained by single cross maize hybrids evaluated at Mississippi State in 2006 and 2007.**

| | Leaf feeding ratings (Mean ± SEM) | | |
|---|---|---|---|
| Genotype | 2006 | 2007 | Mean |
| Mo17 × Tx601 | 9.0 ± 0.0 a | 8.0 ± 0.0 a | 8.5 ± 0.5 ab |
| SC229 × Va35 | 9.0 ± 0.0 a | 8.7 ± 0.6 a | 8.8 ± 0.4 ab |
| Ab24E × Tx601 | 9.0 ± 0.0 a | 8.7 ± 0.6 a | 8.8 ± 0.4 ab |
| SC229 × Tx601 | 9.0 ± 0.0 a | 9.0 ± 0.0 a | 9.0 ± 0.0 a |
| Ab24E × SC229 | 9.0 ± 0.0 a | 8.7 ± 0.6 a | 8.8 ± 0.4 ab |
| Mp488 × SC213 | 9.0 ± 0.0 a | 8.7 ± 0.6 a | 8.8 ± 0.4 ab |
| Ab24E × Va35 | 8.3 ± 0.6 ab | 8.0 ± 0.0 a | 8.2 ± 0.4 b |
| Mp496 × Mp716 | 8.3 ± 1.2 ab | 3.7 ± 1.52 def | 6.0 ± 2.8 c |
| Mp707 × Mp714 | 8.0 ± 0.0 abc | 3.0 ± 0.0 fg | 5.5 ± 2.7 e |
| Mp496 × Mp707 | 8.0 ± 0.0 abc | 3.3 ± 1.2 ef | 5.7 ± 2.7 de |
| Mp703 × Mp704 | 8.0 ± 0.0 abc | 4.0 ± 1.1 cdef | 6.0 ± 2.3 cde |
| Mp707 × Mp708 | 8.0 ± 0.0 abc | 3.7 ± 0.6 def | 5.8 ± 2.4 de |
| Mp704 × Mp713 | 8.0 ± 0.0 abc | 4.7 ± 0.6 cd | 6.3 ± 1.9 cd |
| Mp704 × Mp708 | 7.7 ± 0.6 bcd | 3.7 ± 1.2 def | 5.7 ± 2.3 de |
| Mp713 × Mp97:158 | 7.3 ± 1.2 bcde | 4.3 ± 1.2 cde | 5.8 ± 1.9 de |
| Mp708 × Mp97:158 | 7.3 ± 0.6 bcde | 5.0 ± 1.0 c | 6.2 ± 1.5 cde |
| Mp707 × Mp714 | 8.0 ± 0.0 abc | 3.0 ± 0.0 fg | 5.5 ± 2.7 e |
| Mp704 × Mp707 | 6.7 ± 0.6 aef | 6.7 ± 0.6 b | 6.7 ± 0.5 cd |
| Mp713 × Mp716 | 6.3 ± 0.6 ef | 3.0 ± 1.0 fg | 4.7 ± 2.0 f |
| Mp714 × Mp716 | 6.0 ± 1.7 f | 2.0 ± 0.0 g | 4.0 ± 2.4 f |

Leaf feeding damage was visually rated 14 d after infestation in 2006 and 12 days after infestation in 2007 with 30 fall armyworm neonates per plant on a scale of 0 (no damage) and 9 (extensive damage). Means within a column followed by the same letter do not differ significantly ($P < 0.05$, Fisher's Protected LSD).

2007. The resistant hybrid Mp714 × Mp716 sustained the least damage each year with a mean rating of 4.

Differences among hybrids were significant in 2006 ($F = 5.99$; df = 19, 38; $P < 0.001$). Six hybrids had mean damage ratings of 9.0, and Mp714 × Mp716 sustained the least damage with a mean of 6.0 (Table 1). In 2007 differences among hybrids were significant ($F = 46.73$; df = 19, 38; $P < 0.0001$). Only one hybrid, SC229 × Tx601, rated 9.0, and Mp714 × Mp716 again sustained the least damage with a rating of 2. A rating of 2 indicates that only pin-hole and shot-hole damage was observed on the whorl leaves. All of the hybrids that were produced by crossing lines that had been developed as sources of resistance to insect feeding sustained significantly less damage than the other hybrids in 2007.

**Southwestern corn borer leaf feeding damage evaluations.** As with fall armyworm, southwestern corn borer leaf feeding was heavier in 2006 than in 2007 with an overall mean of 7.2 in 2006 and 5.4 in 2007 (Table 2). Hybrids ($F = 18.68$; df = 19, 76; $P < 0.0001$), years ($F = 21.04$; df = 1, 4; $P < 0.05$), and hybrids × years ($F = 2.33$, df = 19, 76; $P < 0.005$) were significant sources of variation in the combined analysis of variance. As with fall armyworm, Mp714 × Mp716 had the lowest overall level of damage (4.5). Some of the other hybrids, however, did

**Table 2. Southwestern corn borer leaf feeding damage sustained by single cross maize hybrids evaluated at Mississippi State in 20006 and 2007.**

| | Leaf feeding ratings (Mean ± SEM) | | |
|---|---|---|---|
| Genotype | 2006 | 2007 | Mean |
| Mo17 × Tx601 | 8.3 ± 1.2 a | 6.3 ± 0.6 bc | 7.3 ± 1.4 b |
| SC229 × Va35 | 8.0 ± 0.0 a | 7.3 ± 0.6 ab | 7.7 ± 0.5 ab |
| Ab24E × Tx601 | 8.7 ± 0.6 a | 7.7 ± 0.6 a | 8.2 ± 0.8 a |
| SC229 × Tx601 | 8.3 ± 0.6 a | 7.0 ± 0.0 ab | 7.7 ± 0.8 ab |
| Ab24E × SC229 | 8.0 ± 0.0 a | 7.7 ± 0.6 a | 7.8 ± 0.8 ab |
| Mp488 × SC213 | 8.7 ± 0.6 a | 7.7 ± 0.6 a | 8.2 ± 0.8 a |
| Ab24E × Va35 | 8.3 ± 0.6 a | 7.3 ± 0.6 ab | 7.8 ± 0.8 ab |
| Mp496 × Mp716 | 7.0 ± 1.0 abc | 4.7 ± 0.6 def | 5.8 ± 1.5 cd |
| Mp707 × Mp714 | 6.3 ± 1.5 bcd | 4.0 ± 0.0 f | 5.2 ± 1.6 de |
| Mp496 × Mp707 | 7.7 ± 0.6 ab | 4.7 ± 0.6 def | 6.2 ± 1.7 c |
| Mp703 × Mp704 | 7.0 ± 1.0 abc | 5.7 ± 0.6 cd | 6.3 ± 1.0 c |
| Mp707 × Mp708 | 8.0 ± 0.0 a | 4.3 ± 0.6 ef | 6.2 ± 2.0 c |
| Mp704 × Mp713 | 7.3 ± 0.6 ab | 4.3 ± 0.6 ef | 5.8 ± 1.7 cd |
| Mp704 × Mp708 | 6.0 ± 2.6 cd | 4.3 ± 0.6 ef | 5.2 ± 1.9 de |
| Mp713 × Mp97:158 | 6.0 ± 1.0 cd | 4.0 ± 0.0 f | 5.0 ± 1.3 de |
| Mp708 × Mp97:158 | 7.0 ± 1.0 abc | 4.3 ± 0.6 ef | 5.7 ± 1.6 cd |
| Mp707 × Mp714 | 5.7 ± 1.5 cd | 4.3 ± 1.2 ef | 5.0 ± 1.4 de |
| Mp704 × Mp707 | 7.0 ± 0.0 abc | 5.3 ± 1.2 cde | 6.2 ± 1.2 c |
| Mp713 × Mp716 | 5.3 ± 0.6 d | 4.0 ± 0.0 f | 4.7 ± 0.8 e |
| Mp714 × Mp716 | 5.0 ± 1.0 d | 4.0 ± 0.0 f | 4.5 ± 0.8 e |

Leaf feeding damage was visually rated 14 d after infestation with 30 southwestern corn borer neonates per plant on a scale of 0 (no damage) and 9 (extensive damage). Means within a column followed by the same letter do not differ significantly ($P < 0.05$, Fisher's Protected LSD).

not differ significantly from Mp714 × Mp716 in level of damage. The hybrids Ab24E × Tx601 and Mp488 × SC213 sustained the heaviest damage (8.2).

Differences among hybrids were significant in both 2006 ($F = 6.29$; df = 19, 38; $P < 0.0001$) and 2007 ($F = 17.68$; df = 19, 38; $P < 0.0001$). In 2006 Ab24E × Tx601 and Mp488 × SC213 sustained the highest level of damage (8.7), and Mp714 × Mp716 had the lowest damage rating (5.0). In 2007 Ab24E × Tx601, Ab24E × SC229, and Mp488 × SC213 sustained the heaviest damage (7.7), and Mp714 × Mp716, Mp713 × Mp716, and Mp713 × Mp97:158 exhibited the lowest level of damage (4.0).

**Fall armyworm laboratory bioassay.** When fall armyworm larvae fed on diets prepared with lyophilized leaf tissue collected from plants that had been infested with either fall armyworm or southwestern corn borer larvae or on tissue collected from plants that had been infested with neither insect, genotype ($F = 201.09$; df = 6, 79; $P < 0.0001$), infestation treatment ($F = 393.39$; df = 2, 79; $P < 0.0001$), and genotype × treatment ($F = 49.67$; df = 12, 79; $P < 0.0001$) were significant sources of variation in larval weights. When larvae fed on tissue from plants that were not infested with either insect, those that fed on leaf feeding resistant single crosses (Mp496 × Mp707, Mp707 × Mp714, Mp713 × Mp714,

**Table 3. Weights of fall armyworm larvae reared on laboratory diets containing lyophilized leaf tissue collected from resistant or susceptible single cross maize hybrids previously infested with fall armyworm, southwestern corn borer, or not infested.**

| Single cross hybrid | Leaf damage rating[a] | Mean larval wt. ± SEM (mg) | | |
|---|---|---|---|---|
| | | Not infested | Fall armyworm infested | Southwestern corn borer infested |
| Ab24E × SC229 | 8.7 ± 0.6 a | 281 ± 20 a | 70 ± 21 b* | 50 ± 22 b* |
| Ab24E × Tx601 | 8.7 ± 0.6 a | 242 ± 49 b | 44 ± 4 c* | 39 ± 11 bc* |
| SC229 × Tx601 | 9.0 ± 0.0 a | 289 ± 11 a | 189 ± 15 a* | 100 ± 12 a* |
| Mp496 × Mp707 | 3.3 ± 1.2 bc | 32 ± 11 d | 19 ± 2 d | 24 ± 6 c |
| Mp707 × Mp714 | 3.0 ± 0.0 cd | 33 ± 22 d | 20 ± 2 d | 23 ± 5 c |
| Mp713 × Mp716 | 3.0 ± 1.0 cd | 43 ± 15 d | 27 ± 3 cd | 28 ± 5 bc |
| Mp714 × Mp716 | 2.0 ± 0.0 d | 150 ± 18 c | 34 ± 9 cd* | 48 ± 23 b* |

Leaf feeding damage rating and mean weights of fall armyworm larvae reared for 10 d on diets prepared from lyophilized leaf tissue in a column followed by the same letter do not differ significantly ($P < 0.05$, Fisher's Protected LSD). Mean weights of larvae reared on tissue from infested plants in a row followed by an asterisk (*) differ significantly from weights of larvae reared on tissue from non-infested plants.
[a]Visual ratings of damage from fall armyworm feeding rated on a scale of 0 (no damage) to 9 (extensive damage) in 2007 (from Table 1).

Mp714 × Mp716) weighed only about 25% as much as those that fed on the susceptible single crosses (Ab24E × SC229, Ab24E × Tx601, SC229 × Tx601) (Table 3). The mean weights of fall armyworm larvae that fed on tissue from plants infested in the field with fall armyworm, southwestern corn borer, or not infested were 156, 57, and 45 mg, respectively. For the three susceptible hybrids and for Mp714 × Mp716, larvae reared on tissue from infested plants were significantly smaller than those fed on tissue from non-infested plants. For the other three resistant hybrids, larvae reared on non-infested plants were quite small (36 mg). The larvae that fed on diets containing tissue of plants that had been infested weighed less, but the differences were not statistically significant.

**Southwestern corn borer laboratory bioassay.** When southwestern corn borer larvae fed on diets prepared with lyophilized leaf tissue collected from plants that had been infested with either fall armyworm or southwestern corn borer larvae or on tissue collected from plants that had been infested with neither insect, genotype ($F = 25.44.09$; df = 6, 809; $P < 0.0001$), infestation treatment ($F = 52.35$; df = 2, 80; $P < 0.0001$), and the genotype × treatment interaction ($F = 2.44$; df = 12, 80; $P < 0.009$) were significant sources of variation in larval weights. When fed on diets containing tissue of plants that had not been infested with insects, southwestern corn borer larvae that fed on the susceptible hybrids weighed twice as much as those that fed on resistant hybrids (Table 4). Differences among hybrids within the resistant and susceptible groupings were not significant. Larval weights for the susceptible hybrids were significantly lower on diets prepared from tissue subjected to leaf feeding in the field than on diets prepared from non-infested plants. Again, the same trend was observed

**Table 4. Weights of southwestern corn borer larvae reared on laboratory diets containing lyophilized leaf tissue collected from resistant or susceptible single cross maize hybrids previously infested with southwestern corn borer, fall armyworm, or not infested.**

| | | Mean larval wt. ± SEM (mg) | | |
|---|---|---|---|---|
| Single cross hybrid | Leaf damage rating[a] | Not infested | Fall armyworm infested | Southwestern corn borer infested |
| Ab24E × SC229 | 7.7 ± 0.6 a | 77 ± 20 a | 43 ± 8 ab* | 52 ± 10 a* |
| Ab24E × Tx601 | 7.7 ± 0.6 a | 70 ± 25 a | 32 ± 1 bc* | 31 ± 4 b* |
| SC229 × Tx601 | 7.0 ± 0.0 a | 73 ± 28 a | 47 ± 16 *a | 26 ± 5 b* |
| Mp496 × Mp707 | 4.7 ± 0.6 bcd | 30 ± 9 b | 19 ± 7 cd | 17 ± 6 b |
| Mp707 × Mp714 | 4.3 ± 1.2 cd | 35 ± 6 b | 17 ± 2 d* | 19 ± 3 b* |
| Mp713 × Mp716 | 4.0 ± 0.0 d | 30 ± 6 b | 17 ± 3 d | 19 ± 2 b |
| Mp714 × Mp716 | 4.0 ± 0.0 d | 42 ± 6 b | 20 ± 3 cd* | 18 ± 2 b* |

Mean leaf feeding damage rating and weights of southwestern corn borer larvae reared for 14 d on diets prepared from lyophilized leaf tissue in a column followed by the same letter do not differ significantly ($P < 0.05$, Fisher's Protected LSD). Mean weights in a row followed by an asterisk (*) differ significantly from weights of larvae reared on tissue from non-infested plants.

[a]Visual ratings of damage from southwestern corn borer feeding rated on a scale of 0 (no damage) to 9 (extensive damage) in 2007 (from Table 2).

with the resistant hybrids, but the differences were significant for only Mp704 × Mp714 and Mp714 × Mp716.

**Effects of genotype and leaf feeding damage on larval growth in laboratory bioassays.** Of the single cross hybrids selected for inclusion in laboratory bioassays, three had sustained heavy leaf feeding damage and the other four, only moderate damage, by both insects in the 2007 field tests. Both fall armyworm and southwestern corn borer larvae weighed significantly more when fed on tissue of non-infested susceptible genotypes than on non-infested resistant genotypes (Table 3, 4). Although fall armyworm larvae were much larger than southwestern corn borer larvae at the conclusion of the bioassays, their responses to susceptible and resistant genotypes were similar. This appears to indicate that larval growth of both fall armyworm and southwestern corn borer is affected, at least in part, by constitutive differences between resistant and susceptible genotypes at the time the tissue was harvested for bioassays. This would be consistent with a lower percentage of protein and higher percentage of hemicellulose in the leaves of resistant plants (Hedin et al. 1996, Williams et al. 1998), the thicker upper and lower cell wall complex of resistant genotypes (Davis 1995), or even the presence or absence of constitutive defensive proteins. Defenses that cause differences in rate of larval growth may also change with development and maturation of the corn plant (Williams et al. 1998, Chang et al. 2000). This is consistent with the findings that resistance manifested as reduced larval growth is most apparent when insects are fed on the yellow-green portion of leaves and the association between larval growth and juvenile-adult phase transition.

Both fall armyworm and southwestern corn borer larvae reared on the three most susceptible single crosses (Ab24E × SC229, Ab24E × Tx601, SC229 × Tx601) weighed significantly less when fed on tissue from plants that had been infested before the tissue was harvested (Tables 3, 4). This indicates that larval feeding may have induced defensive compounds. A similar trend was also observed with the four more resistant single crosses although the differences were not statistically significant for all single crosses. This may have been a result of the much lower larval weights on the non-infested resistant genotype than on the non-infested susceptible genotypes. Also, if the induction of defensive compounds is related to degree of damage to the host plant, the heavier leaf feeding damage sustained by the susceptible genotypes may have contributed to a greater mobilization of defenses in these genotypes. While it is possible that defensive compounds may have been induced by wounding as tissue was removed from the plants, this would have required a rapid mobilization of defenses because of the procedures followed in harvesting and processing tissue. Relatively large amounts of the Mir 1-CP, a defensive cysteine protease, was found in the non-infested resistant genotypes by Pechan et al. (2000) who suggested that this may have resulted from wounding during the harvesting and processing of the tissue.

It seems reasonable that both constitutive and induced defenses are operating against both fall armyworm and southwestern corn borer in genotypes resistant to leaf feeding damage. It also appears that the defensive compounds are induced in the susceptible corn genotypes as well. Additional experimentation with both leaf feeding susceptible and resistant corn genotypes from the time of infestation to 12 d after infestation may provide a better understanding of defenses against fall armyworm and southwestern corn borer.

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# Fall Armyworm (Lepidoptera: Noctuidae) and Southwestern Corn Borer (Lepidoptera: Crambidae) Leaf Feeding Damage and Its Effect on Larval Growth on Diets Prepared from Lyophilized Corn Leaves[1]

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J. Agric. Urban Entomol. 25(1): 1–11 (January 2008)

**ABSTRACT** Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and Southwestern corn borer, *Diatraea grandiosella* Dyar, are economically important pests of maize (*Zea mays* L.) in the southeastern United States. These insects attack plants in both the vegetative and reproductive stages of growth. Plant resistance is widely considered a desirable means for reducing losses to both insects, and corn germplasm lines with resistance to leaf feeding damage have been developed and released. Fall armyworm and southwestern corn borer larvae feeding on resistant genotypes grow more slowly than those feeding on susceptible genotypes. The objectives of the investigation were to evaluate 20 single cross maize hybrids for leaf feeding damage by southwestern corn borer and fall armyworm, to compare larval growth on laboratory diets prepared from lyophilized leaf tissue of single cross hybrids with different levels of resistance to leaf feeding, and to determine whether larval growth differed between diets prepared from leaf tissue collected from plants previously infested with southwestern corn borer or fall armyworm and non-infested plants. Both fall armyworm and southwestern corn borer larvae weighed significantly less when fed on laboratory diets prepared from lyophilized leaf tissue of resistant single cross hybrids than susceptible hybrids. When tissue from either resistant or susceptible plants that had been infested with either insect in the field was used in bioassays, larval weights were further reduced. It appears that both constitutive and induced defensive mechanisms may be operating.

**KEY WORDS** *Diatraea grandiosella*, *Spodoptera frugiperda*, *Zea mays*, Lepidoptera, Crambidae, Noctuidae, host plant resistance

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith), and Southwestern corn borer, *Diatraea grandiosella* Dyar, are economically important pests of maize (*Zea mays* L.) in the southeastern United States. These insects attack plants in both the vegetative and reproductive stages of growth. Plant resistance is widely considered a desirable means for reducing losses to both insects. Breeding programs have been established at several locations to identify and develop maize germplasm with resistance to these and other Lepidoptera (Smith et al. 1989,

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# Gall Midges (Diptera: Cecidomyiidae) In Forest Trees of Turkey[1]

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**ABSTRACT** The family Cecidomyiidae is one of the largest in the order Diptera. Gall midges are small, inconspicuous flies, but they may be very important both in forest ecosystems and in agroecosystems. The presently known fauna of gall midges in Turkey includes 71 species belonging to 38 genera. Forest gall midge (Diptera: Cecidomyiidae) fauna of Turkey was examined to determine that the composition consists of 33 species in 24 genera from 34 host tree species representing 15 tree genera. Of these, 31 species are phytophagous, 1 species is zoophagous, and 1 species is phytosaprophagous.

**KEY WORDS** Gall midges, Cecidomyiiade, Diptera, forest trees, fauna, Turkey

## Introduction

The family Cecidomyiidae is one of the largest in the order Diptera. Gall midges are small, inconspicuous flies, but they may be very important both in forest ecosystems and in agroecosystems. Many phytophagous gall midge species attack forest trees, and some of them can be serious pests, such as the *Dasineura rozhkovii* Mamaev and Nikolsky, which develops in bud galls of *Larix sibiriea* Ledeb (Isaev et al. 1988). More than 1200 species in 125 genera are known to occur in the Nearctic Region (Stone et al. 1965), and about 2200 species in 300 genera occur in the Palearctic Region (Skuhravá 1986). It has been estimated that the world fauna of gall midges includes four or five thousand species.

Gall midges are very fragile small insects, usually only 0.5 to 3 mm long, rarely up to 8 mm long. They are characterized by hairy wings, considered unusual in the order Diptera, and possess long antennae. Adults do not take food and therefore cannot cause damage to humans in any way. The larvae of gall midges feed and develop in various environments.

On the basis of larval feeding habits, gall midges may be divided into three ecological groups: the mycophagous (=fungivorous), the phytophagous, and the zoophagous (Skuhravá et al. 1984). The larvae of phytophagous gall midges develop in, or on, various organs of many host plant species, where they feed by sucking sap from the tissue. The family Cecidomyiidae have many species that

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intergrades between gall making and leaf mining. These midges form distinctive galls on different plants.

Barnes (1951) has reviewed about 240 gall midge species associated with coniferous and broad-leaved trees in the world. The larvae of 80 species develop on various species of 14 genera of coniferous trees, making galls on buds, terminal shoots, needles, fruits, seeds, and cones, or living under bark, in resin masses, in scolytid burrows, and in aphid galls. In Europe only seven species may be considered serious pests: *Therodiplosis brachyptera* Schwagr on needles of *Pinus silvestris* L. and *P. mugo* Turra, *Resseliella piceae* Seitner on seed of *Abies alba* Mill., *Plemeliella abietina* Seitner on seeds of *Picea aceba* Link., *Dasineura kellneri* Henschel (=*Dasineura larick* Low 1878) on buds of *Larix decidua* Mill., *Paradiplosis abietis* Hubault on needles of *Abies alba* Mill, and *Janetiella skkiyou* Felt (=*Carneva lawsoniana* de Meijere) on the seeds of *Chamaecyparis lawsoniana* Parl imported from North America. The larvae of about 160 gall midge species develop on various species of 35 genera of broad-leaved trees in the world, producing various types of galls on terminal buds, stems, twigs, leaves, petioles, flowers, flower-stalks, fruits, and pods, or living under the bark, in decaying wood and bark, or as predators in the galls of mites or aphids (Skuhravá et al. 1991).

Trotter (1903) was the first to collect galls on forest trees in Turkey. He identified 7 gall midge species: *Dryomyia circinnans*, *Hartigiola annulipes*, *Janetia cerris*, *Macrodiplosis pustularis*, *Macrodiplosis roboris*, *Mikiola fagi*, *Oligotrophus panteli*, and *Zygiobia carpini* occurring in Turkey. Other observations include: *Clinodiplosis botularia* (Acatay 1943), five gall midge species from forest trees (Alkan 1952) and several others described from Turkish forests (Schimitschek 1953, Defne 1954, Bodenheimer 1958, Acatay 1959, Thomson & Simmonds 1965, Ural & Kurt 1971). In 1975, Toros & Kılınçer added the species *Monarthropalpus flavus*. Sarıoğlu (1976), Kurt (1982) and Özay (1997) provided a list of species in forest ecosystems existing in Turkey. During 2000–2001, three gall midge species observed in samples of cones from *Pinus brutia*, *viz. Asynapta strobi*, *Camptomyia pinicola* and *Thecodiplosis* sp. have been reared and identified (Can 2003). The purpose of this study was to update and assimilate all known records of gall midges from Turkey to provide the most current list of species known to date.

## Methods

A checklist of gall midges (Diptera: Cecidomyiiade) was constructed from published records taken by Skuhravá et al. (2005) to determined gall midges presently found in forests of Turkey (Fig. 1). For each species the following data were given: biology, the host plant species and plant family, references and distribution. Synonyms which occurred in the Turkish literature in the past were also provided.

## Results and Discussion

This study represents the most recent and comprehensive assimilation of literature concerning forest gall midge (Diptera: Cecidomyiidae) fauna of Turkey (Table 1). Thirty-three species from 24 genera belonging to Cecidomyiidae in

forest trees are reported from Turkey (Table 2). Of these, 31 species are phytophagous, 1 species is zoophagous, and 1 species is phytosaprophagous.

The gall midge species in forest trees of Turkey may be divided into six groups: European, Euro-Sibarian, Mediterranean, Sub-Mediterranean, West-Asian, and Southwest-Asian. Sixteen species (49%) of these are European: *Arthrocnodax coryligallarum* on *Corylus avellana L.*, *Macrodiplosis pustularis* and *Macrodiplosis roboris* on *Quercus* sp., *Hartigiola annulipes* and *Mikiola fagi* on *Fagus* sp., *Resseliella piceae* on *Abies alba*, *Zygiobia carpini* on *Carpinus betulus*, *Janetiella lemeei* on *Ulmus minor*, *Monarthropalpus flavus* on *Buxus sempervinens*, *Oligotrophus juniperinus* and *Oligotrophus panteli* on *Juniperus* sp., *Physemocecis hartigi* on *Tilia* sp., *Asynapta strobi* on *Pinus brutia*, *Picea excelsa* and *Picea abies*, *Camptomyia pinicola* on *Pinus* sp. Fourteen species are phytophagous, one species is zoophagous and one species is phytosaprophagous. Eleven species (33%) occurring in Turkey may be classified as Euro-Siberian: *Contarinia coryli* on *Corylus avellana*, *Contarinia populi*, *Harmandiola cavernosa*, *Harmandiola globuli* and *Lasioptera populnea* on *Populus tremula*, *Iteomyia capreae*, *Rabdophaga heterobia*, *Rabdophaga rosaria*, *Rabdophaga saliciperda*, *Rabdophaga salicis* and *Rabdophaga terminalis* on *Salix* sp. All of these species are phytophagous. Two species (6%) occurring in Turkey may be classified as West-Asian: *Mikiola orientalis* on *Fagus* sp. and *Thecodiplosis* sp. on *Pinus brutia*. Two species in this group are phytophagous. Two species (6%) occurring in Turkey may be classified as Mediterranean: *Janetia cerris* and *Janetia szepligetii* on *Quercus cerris*; two species in this group are phytophagous. One species (3%) occurring in Turkey may be classified as Southwest Asian: *Taxomyia taxi* on *Taxus baccata*. This species is phytophagous; one species (3%) occurring in Turkey may be classified as Sub-Mediterranean: *Dryomyia circinans* on *Quercus cerris*. This species is also phytophagous.

The forest gall midges species fauna of Turkey is not distributed regularly over the entire territory of Turkey. Twenty-one species (63%) were found in the Marmara Region. This region is among the richest in Turkey for gall midges occurring in forests. Other locations include the internal Anatolian Region, 8 species (24%); Black Sea Region 7 species (21%); Aegean Region, 6 species (18%). In the Mediterranean, East Anatolian and South East Anatolian Regions no species have been recorded.

A list of forest gall midges of Turkey are provided herein:

***Arthrocnodax coryligallarum*** (Targioni-Tozzetti, 1887)

Larvae are predators of eriophyid mites *Phytoptus avellanae* Nal. (Acarina: Eriophyoidea) in big bud galls on *Corylus avellana* L. (Corylaceae) (Skuhravá, 1994b). Distribution: European. Catalogued from Turkey without giving the name of the locality (Thomson & Simmonds, 1965).

***Asynapta strobi*** (Kieffer, 1920)

One female was reared from cones of *Pinus brutia* Ten. (Pinaceae) on 10.X.2001 in Izmir (Kınık) by Can (2003). Larvae of *A. strobi* are known to develop in cones of *Picea abies* (L.) Karst. [=*Picea excelsa* (Lam.) Link.] and probably also in cones of other species of Pinaceae. Larvae are not phytophagous, they are phytosaprophagous. Distribution: European. Izmir (Kınık) (Can 2003).

***Camptomyia pinicola*** Mamaev, 1961

Larvae were originally found as developing under the bark of *Pinus sylvestris* L. (Pinaceae) in Russia. Roques (1983) found this species in cones of *Pinus*

*sylvestris* and *P. halepensis* Mill. at several places in France. He found that two generations of gall midges develop in cones of *Pinus sylvestris*. Can (2003) reared adults from cones of *Pinus brutia* Ten. from three localities in Turkey during 2000 and 2001. Adults reared in Manisa (Muradiye), on 4.-17.V.2000; 2.-8.XI.2000; 5.IV.-27.V.2001; 2.X.-15.XI.2001; in Manisa (Gelenbe), on 17.IV. 15.V.2000; 17.IV.-15.V.2001; in Izmir (Kınık), on 8.-20.IV.2000; 3.IX.-14.X.2000; 17.IV.-12.V.2001; 10.IX.-12.X.2001.

Distribution: European. Manisa (Muradiye, Gelenbe), Izmir (Kınık). (Can, 2003).

***Clinodiplosis botularia*** (Winnertz, 1853)

Larvae live in galls on the main vein of the leaf of *Fraxinus excelsior* L. (Oleaceae). Distribution: European (Skuhravá 1997b). Reported from Istanbul (Acatay, 1943).

***Contarinia coryli*** (Kaltenbach, 1859)

Synonym: *Diplosis corylina* F. Löw, 1878, Larvae develop in swollen catkins of *Corylus avellana* L. (Corylaceae) (Skuhravá 1994b). Distribution: Euro-Siberian (Skuhravá, 1997b). Reported from the East Black Sea Region in Turkey (Sarıoğlu, 1976; Kurt, 1982).

***Contarinia populi*** (Rübsaamen, 1917)

Larvae cause small rounded galls on leaves of *Populus tremula* L. (Salicaceae). In galls of *C. populi* the larvae of *Lasioptera populnea* live as inquilines. Distribution: Euro-Siberian (Skuhravá, 1997b). Reported from Istanbul (Belgrad Forest, Alemdağ) and Balıkesir (Dursunbey) under the name of ***Diplosis populi*** (Rübsaamen) (Acatay, 1959).

***Dryomyia circinans*** (Giraud, 1861)

Larvae cause hard galls on leaves of *Quercus cerris* L. (Fagaceae) (Skuhravá, 1994b). Distribution: Sub-Mediterranean and Mediterranean (Skuhravá, 1986). Reported from Bilecik (Trotter, 1903), Istanbul on *Q. pubescens* Wil. (Acatay, 1943), Eskişehir (Sivrihisar) on *Quercus* sp., Manisa (Salihli, Alaşehir), Niğde (Hasandağı) (Karaca 1956).

***Harmandiola cavernosa*** (Rübsaamen, 1899)

Larvae cause large, thick walled galls on leaves of *Populus tremula* L. (Salicaceae) with an opening on the upper surface (Skuhravá 1994b). Distribution: Euro-Siberian (Skuhravá, 1997b). Reported from Istanbul (Belgrad Forest, Alemdağ) and Balıkesir (Dursunbey) (Acatay 1959).

***Harmandiola globuli*** (Rübsaamen, 1889)

Larvae cause small, rounded and thin walled galls on leaves of *Populus tremula* L. (Salicaceae) with an opening on the lower leaf surface (Skuhravá 1994b). Distribution: Euro-Siberian (Skuhravá 1997b). Reported from Istanbul (Belgrad Forest, Alemdağ) and Balıkesir (Dursunbey) (Acatay 1959).

***Hartigiola annulipes*** (Hartig, 1839)

Synonym: *Phegobia tornatella* Bremi, 1847. Larvae cause galls on leaves of *Fagus sylvatica* L. (Fagaceae) (Skuhravá 1994b). Distribution: European (Skuhravá 1997b). Reported from Adapazarı (Sapanca) on *F. orientalis* as *Oligotrophus* sp. (Trotter, 1903), Bolu (Abant) (Alkan 1952), Istanbul on *Fagus* sp. (Acatay 1943, Schimitschek, 1953).

***Iteomyia capreae*** (Winnertz, 1853)

Larvae cause small globular galls on leaves of *Salix caprea* L. (Salicaeae) (Skuhravá 1994b). Distribution: Euro-Siberian (Skuhravá 1997b). Reported from Turkey without exact locality (Bodenheimer 1958).

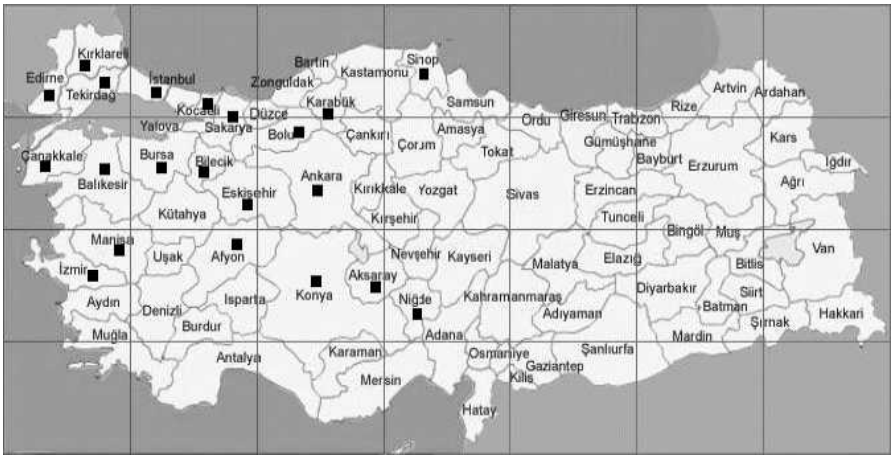


**Fig. 1.** The distribution of gall midges in forest trees according to provinces of Turkey. Small squares depicted represent locations of published accounts of gall midge species in Turkey.

***Janetia cerris*** (Kollar, 1850)

Larvae cause small hard galls on leaves of *Quercus cerris* L. (Fagaceae) (Skuhravá 1994b). Distribution: Mediterranean (Skuhravá 1997b). Cited from Bursa (Gemlik Iznik) as *Arnoldia* sp. (Trotter 1903) and from Aksaray (Hasandağı), Izmir (Menemen) on *Quercus* spp. under the name of *Arnoldia cerris* Kollar (Karaca 1956).

***Janetia szepligetii*** (Kieffer, 1896)

Larvae cause small pustule galls on leaves of *Quercus cerris* L. (Fagaceae) (Skuhravá 1994b). Distribution: Mediterranean (Skuhravá 1997b). Recorded from Adapazarı (Sapanca), Bursa (Trotter 1903).

***Janetiella lemeei*** (Kieffer, 1904)

Larvae cause small galls on leaves of *Ulmus minor* Mill and other species (Ulmaceae) (Skuhravá 1994b). Distribution: European (Skuhravá, 1997b). Cited from Ankara (Alkan, 1952).

***Lasioptera populnea*** Wachhtl, 1883

Larvae live as inquilines in galls caused by *Contarinia populi* on leaves of *Populus tremula* L. (Salicaceae) (Skuhravá 1994b). Distribution: Euro-Siberian. Reported from Istanbul (Belgrad Forest, Alemdağ) and Balıkesir (Dursunbey) (Acatay, 1959).

***Macrodiplosis pustularis*** (Bremi, 1847)

**S**ynonym: *Macrodiplosis dryobia* (F. Löw, 1877). Larvae cause leaf galls in the form of folded leaf lobe downwards of *Quercus robur* L. (Fagaceae) (Skuhravá 1994b). Distribution: European (Skuhravá 1997b). Reported from Bursa on *Quercus lusitanica* Lm., Sakarya (Adapazarı, Sapanca) on *Quercus petraea* (Matt.) Liebl. (=*Q. sessiflora* Salis.) (Trotter 1903).

***Macrodiplosis roboris*** (Hardy, 1854)

Synonym: *Macrodiplosis volvens* Kieffer, 1895. Larvae cause leaf galls (rolled leaf margin upwards) of *Quercus robur* L. (Fagaceae) (Skuhravá 1994b). Distribution: European (Skuhravá 1997b). Reported from Bursa (Gemlik) on

**Table 1. Distribution of gall midges species according to region of Turkey.**

| Species Number | Species name | Black Sea Region | Marmara Region | Aegean Region | Mediterranean Region | Internal Anatolian Region | East Anatolian Region | Southeast Anatolian Region |
|---|---|---|---|---|---|---|---|---|
| 1 | ***Arthrocnodax coryligallarum*** (Targioni-Tozzetti) | **Reported from Turkey without exact locality** | | | | | | |
| 2 | ***Asynapta strobi*** (Kieffer) | – | – | + | – | – | – | – |
| 3 | ***Camptomyia pinicola*** (Mamamev) | – | – | + | – | – | – | – |
| 4 | ***Clinodiplosis botularia*** (Winnertz) | – | + | – | – | – | – | – |
| 5 | ***Contarinia coryli*** (Kaltenbach) | + | – | – | – | – | – | – |
| 6 | ***Contarinia populi*** (Rübsaamen) | – | + | – | – | – | – | – |
| 7 | ***Dryomyia circinans*** (Giraud) | – | + | + | – | + | – | – |
| 8 | ***Harmandiola cavernosa*** (Rübsaamen) | – | + | – | – | – | – | – |
| 9 | ***Harmandiola globuli*** (Rübsaamen) | – | + | – | – | – | – | – |
| 10 | ***Hartigiola annulipes*** (Hartig) | + | + | – | – | – | – | – |
| 11 | ***Iteomyia capreae*** (Winnertz) | **Reported from Turkey without exact locality** | | | | | | |
| 12 | ***Janetia cerris*** (Kollar) | – | + | + | – | + | – | – |
| 13 | ***Janetia szepligetii*** (Kieffer) | – | + | – | – | – | – | – |
| 14 | ***Janetiella lemeei*** (Kieffer) | – | – | – | – | + | – | – |
| 15 | ***Lasioptera populnea*** (Wachhtl) | – | + | – | – | – | – | – |
| 16 | ***Macrodiplosis pustularis*** (Bremi) | – | + | – | – | – | – | – |
| 17 | ***Marcodiplosis roboris*** (Hardy) | – | + | – | – | – | – | – |
| 18 | ***Mikiola fagi*** (Hartig) | + | + | – | – | – | – | – |
| 19 | ***Mikiola orientalis*** (Kieffer) | – | + | – | – | – | – | – |
| 20 | ***Mikomya coryli*** (Kieffer) | + | – | – | – | – | – | – |
| 21 | ***Monarthropalpus flavus*** (Schrank) | – | – | – | – | + | – | – |
| 22 | ***Oligotrophus juniperinus*** (Linnaeus) | – | + | – | – | – | – | – |
| 23 | ***Oligotrophus panteli*** (Kieffer) | – | + | – | – | – | – | – |
| 24 | ***Physemocecis hartigi*** (Liebel) | – | + | – | – | – | – | – |

**Table 1. Continued.**

| Species Number | Species name | Black Sea Region | Marmara Region | Aegean Region | Mediterranean Region | Internal Anatolian Region | East Anatolian Region | Southeast Anatolian Region |
|---|---|---|---|---|---|---|---|---|
| **25** | ***Rabdophaga heterobia*** (Loew) | – | – | – | – | + | – | – |
| **26** | ***Rabdophaga rosaria*** (Low) | + | + | – | – | + | – | – |
| **27** | **Rabdophaga *saliciperda*** (Dufour) | – | + | + | – | + | – | – |
| **28** | ***Rabdophaga salicis*** (Schrank) | – | + | | – | + | – | – |
| **29** | ***Rapdophaga terminalis***(Loew) | – | + | – | – | – | – | – |
| **30** | ***Resseliella piceae*** (Seitner) | + | – | – | – | – | – | – |
| **31** | ***Taxomyia taxi*** (Inchbald) | + | – | – | – | – | – | – |
| **32** | ***Thecodiplosis sp.*** | – | – | + | – | – | – | – |
| **33** | ***Zygiobia carpini*** (F. Löw) | – | + | – | – | – | – | – |

*Quercus lusitanica* Lm., Sakarya (Adapazarı, Sapanca) on *Quercus sessiflora* Sm. (Trotter 1903), Istanbul on *Quercus* sp. (Alkan 1952).

***Mikiola fagi*** (Hartig, 1839)

Larvae cause hard pointed galls on the upper leaf side of *Fagus sylvatica* L. (Fagaceae) (Skuhravá 1994b). Distribution: European (Skuhravá, 1997b). Reported from Adapazarı (Sapanca) (Trotter, 1903), Istanbul (Acatay 1943), Bolu (Abant) on *Fagus* sp. (Alkan 1952).

***Mikiola orientalis*** Kieffer, 1908

Larvae cause large hard pointed galls on upper leaf side of *Fagus orientalis* L. Distribution: West-Asian (Skuhravá 1986). Recorded from Istanbul on *Fagus* sp. (Schimitschek 1953).

***Mikomya coryli*** (Kieffer, 1901)

Larvae cause small galls (depressions) on leaves of *Corylus avellana* L. (Corylaceae) (Skuhravá 1994b). Distribution: European (Skuhravá 1997b). Reported from Eastern Black Sea Region (Ural & Kurt 1971, Kurt 1973).

***Monarthropalpus flavus*** (Schrank, 1776)

Synonym: *Monarthropalpus buxi* Laboulbéne, 1873. Larvae cause small blister galls which are apparent on both sides of leaves of *Buxus sempervirens* L. (Buxaceae) (Skuhravá 1994b). Distribution: European, secondarily Holarctic (Skuhravá 1986). Cited from Ankara (Toros & Kılınçer 1975).

***Oligotrophus juniperinus*** (Linnaeus, 1758)

Larvae cause galls on terminal or lateral buds of *Juniperus communis* L. (Cupressaceae) (Skuhravá & Skuhravy 1997). Distribution: European (Skuhravá & Skuhravy 1997). Cited from Istanbul (Schimitschek 1953).

***Oligotrophus panteli*** Kieffer, 1898

Larvae cause galls on terminal or lateral buds of *Juniperus juniperinus* L. and *J. oxycedrus* L. (Cupressaceae). Distribution: European (Skuhravá & Skuhravy 1997). Reported from Bursa (Mudanya) (Trotter 1903).

***Physemocecis hartigi*** (Liebel, 1892)

Larvae cause rounded blister galls on leaves of *Tilia* spp. (Tiliaceae) (Skuhravá 1994b). Distribution: European (Skuhravá 1997b). Cited from Istanbul (Alkan 1952).

***Rabdophaga heterobia*** (Loew, 1850)

Larvae of hibernation generation cause swellings on catkins and larvae of summer generation large leaf rosettes, densely haired, on *Salix triandra* L. (Salicaceae). Distribution: Euro-Siberian (Skuhravá 1997b). Cited from Ankara (Kalecik) on *Salix* sp. (Alkan 1952).

***Rabdophaga rosaria*** (*Low*, 1850)

Larvae cause large rosette galls on *S. alba* (Skuhravá & Skuhravy 1997). Distribution: Euro-Siberian (Skuhravá & Skuhravy 1997). Reported from Ankara, Eskişehir (Sivrihisar), Konya, Niğde on *Salix alba* and *S. purpurea* L. (Alkan 1952; Karaca 1956); Karabük (Kurşunlu) (Acatay 1971); Kırklareli (Demirköy, Longoz), Edirne (Uzunköprü, Keşan), Sakarya (Adapazarı, Karasu), Kocaeli (Izmit, Maşukiye) on *Salix alba* (Özay 1997).

***Rabdophaga saliciperda*** (Dufour, 1841)

Larvae develop under the bark of *Salix fragilis* L. and *S. alba*. Attacked branch is swollen with many emergence openings. It was also reported under the name of *Helicomyia saliciperda* (Dufour). Distribution: Euro-Siberian (Skuhravá 1997b). Reported from Ankara (Haymana) (Alkan 1952), Western Anatolia on

**Table 2. Distribution of gall midges species according to host forest trees from Turkey.**

| Gall Midge Species | Host(s) Tree(s) |
|---|---|
| 1. *Arthrocnodax coryligallarum* | *Phytoptus avellanae*, *Corylus avellana L.* |
| 2. *Asynapta strobi* | *Pinus brutia*, *Picea abies* , *Picea excelsa* |
| **3.** *Camptomyia pinicola* | *Pinus sylvestris* L., *P. halepensis* |
| 4. *Clinodiplosis botularia* | *Fraxinus excelsior* L. |
| 5. *Contarinia coryli* | *Corylus avellana* L. |
| 6. *Contarinia populi* | *Populus tremula* L. |
| 7. *Dryomyia circinans* | *Quercus cerris* L., *Q. pubescens* |
| 8. *Harmandiola cavernosa* | *Populus tremula* L. |
| 9. *Harmandiola globuli* | *Populus tremula* L. |
| 10. *Hartigiola annulipes* | *Fagus sylvatica* L., *F. orientalis*, *Oligotrophus* sp. |
| 11. *Iteomyia capreae* | *Salix caprea* L. |
| 12. *Janetia cerris* | *Quercus cerris* L., |
| 13. *Janetia szepligetii* | *Quercus cerris* L. |
| 14. *Janetiella lemeei* | *Ulmus minor* |
| 15. *Lasioptera populnea* | *Contarinia populi* |
| 16. *Macrodiplosis pustularis* | *Quercus robur* L., *Q. lusitanica*, *Q. petraea* (=*Q. sessiflora Salis.*) |
| 17. *Marcodiplosis roboris* | *Quercus robur* L., *Q. lusitanica*, *Q. sessiflora* |
| 18. *Mikiola fagi* | *Fagus sylvatica* L. |
| 19. *Mikiola orientalis* | *Fagus orientalis* L. |
| 20. *Mikomya coryli* | *Corylus avellana* L. |
| 21. *Monarthropalpus flavus* | *Buxus sempervirens* L. |
| 22. *Oligotrophus juniperinus* | *Juniperus communis* L. |
| 23. *Oligotrophus panteli* | *Juniperus juniperinus* L., *J. oxycedrus* L. |
| 24. *Physemocecis hartigi* | *Tilia* spp. |
| 25. *Rabdophaga heterobia* | *Salix triandra* L. |
| 26. *Rabdophaga rosaria* | *Salix alba*, *S. purpurea L.* |
| 27. *Rabdophaga saliciperda* | *Salix fragilis* L., *S. alba* |
| 28. *Rabdophaga salicis* | *Salix aurita* L, *S. alba*, *S. babylonica*, *S. excelsa* |
| 29. *Rapdophaga terminalis* | *Salix fragilis*, *S. alba*, *Salix excelsa* |
| 30. *Resseliella piceae* | *Abies alba* |
| 31. *Taxomyia taxi* | *Taxus baccata* L. |
| 32. *Thecodiplosis* sp. | *Pinus brutia* |
| 33. *Zygiobia carpini* | *Carpinus betulus* L. |

*Salix* sp. (Yargıç & Türkmenoğlu 1948), Afyon (Dinar), Konya, Eskişehir (Acatay 1971); Sakarya (Adapazarı, Geyve, Ferizli), Kocaeli (Izmit), Bursa (Mustafakemalpaşa, Inegöl), Bilecik, Balıkesir (Manyas, Susurluk), Çanakkale (Biga, Çan), Edirne(Uzunköprü, Keşan), Kırklareli (Vize, Demirköy) on *S. alba*; Sakarya (Adapazarı, Geyve, Ferizli) on *S. triandra*; Kocaeli (Izmit), Balıkesir (Manyas), Edirne, Istanbul (Bahçeköy) on *S. babylonica* L.; Kocaeli (Izmit) on *S. excelsa* J. F. Gmelin; Sakarya (Adapazarı, Ferizli) on *S. cinerea* L. (Özay 1997).

***Rabdophaga salicis*** (Schrank, 1803)

Larvae cause globular or cylindrical galls on branches of *Salix aurita* L. and related species of *Salix* (Salicaceae). Distribution: Euro-Siberian (Skuhravá 1997b). Reported from Ankara on *S. alba* (Alkan 1952; Bodenheimer 1958); Kocaeli (Izmit),

Sakarya (Adapazarı, Geyve), Bilecik (Küplü), Bursa (Inegöl), Balıkesir (Manyas, Dursunbey, Susurluk, Sındırgı), Istanbul (Bahçeköy), Tekirdağ (Saray), Kırklareli (Vize, Demirköy), Edirne (Uzunköprü, Keşan) on *S. alba*; Kocaeli (Izmit), Edirne on *S. babylonica*; Kocaeli (Izmit) on *S. excelsa* (Özay 1997).

***Rabdophaga terminalis*** (Loew, 1850)

Larvae cause spindle galls on terminal shoots of *Salix fragilis*, *S. alba* and related *Salix*-species. Several generations develop in a year. Distribution: Euro-Siberian (Skuhravá 1997b). Reported from Edirne (Uzunköprü, Keşan), Kırklareli, Tekirdağ (Saray), Bursa (Inegöl), Sakarya (Adapazarı, Söğütlü, Karasu), Bursa (Inegöl, Bilecik, Küplü), Balıkesir (Manyas) on *S. alba*; Sakarya (Adapazarı, Karasu) on *Salix excelsa* (Özay 1997).

***Resseliella piceae*** Seitner, 1906

Larvae develop inside the seed in cones of *Abies alba* Mill. (Pinaceae) (Skuhravá 1994b). Distribution: European (Skuhravá 1997b). Cited from Western Black Sea Region on *Abies* sp. (Defne 1954).

***Taxomyia taxi*** (Inchbald, 1861)

Larvae cause large artichoke-shaped galls on branches of *Taxus baccata* L. (Taxaceae). Distribution: European and southwest-Asian. Reported from Sinop (Ayancık) (Schimitschek 1953).

***Thecodiplosis* sp.**

One male and one female were reared from young cones of *Pinus brutia* Ten. (Pinaceae) on 4.IX.2001 by P. Can. Probably they belong to a new species for science. The status of this species remains doubtful until a more extensive evaluation can be performed. Distribution: West-Asian. Recorded from Manisa (Muradiye) and Izmir (Kınık).

***Zygiobia carpini*** (F. Löw, 1874)

Larvae cause swellings on the midvein of leaves on *Carpinus betulus* L. (Corylaceae) (Skuhravá, 1994b). Distribution: European (Skuhravá 1997b). Reported from Sakarya (Adapazarı, Sapanca) (Trotter 1903) and from Turkey without exact locality (Bodenheimer 1958).

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# Gall Midges (Diptera: Cecidomyiidae) In Forest Trees of Turkey[1]

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**ABSTRACT** The family Cecidomyiidae is one of the largest in the order Diptera. Gall midges are small, inconspicuous flies, but they may be very important both in forest ecosystems and in agroecosystems. The presently known fauna of gall midges in Turkey includes 71 species belonging to 38 genera. Forest gall midge (Diptera: Cecidomyiidae) fauna of Turkey was examined to determine that the composition consists of 33 species in 24 genera from 34 host tree species representing 15 tree genera. Of these, 31 species are phytophagous, 1 species is zoophagous, and 1 species is phytosaprophagous.

**KEY WORDS** Gall midges, Cecidomyiiade, Diptera, forest trees, fauna, Turkey

## Introduction

The family Cecidomyiidae is one of the largest in the order Diptera. Gall midges are small, inconspicuous flies, but they may be very important both in forest ecosystems and in agroecosystems. Many phytophagous gall midge species attack forest trees, and some of them can be serious pests, such as the *Dasineura rozhkovii* Mamaev and Nikolsky, which develops in bud galls of *Larix sibiriea* Ledeb (Isaev et al. 1988). More than 1200 species in 125 genera are known to occur in the Nearctic Region (Stone et al. 1965), and about 2200 species in 300 genera occur in the Palearctic Region (Skuhravá 1986). It has been estimated that the world fauna of gall midges includes four or five thousand species.

Gall midges are very fragile small insects, usually only 0.5 to 3 mm long, rarely up to 8 mm long. They are characterized by hairy wings, considered unusual in the order Diptera, and possess long antennae. Adults do not take food and therefore cannot cause damage to humans in any way. The larvae of gall midges feed and develop in various environments.

On the basis of larval feeding habits, gall midges may be divided into three ecological groups: the mycophagous (=fungivorous), the phytophagous, and the zoophagous (Skuhravá et al. 1984). The larvae of phytophagous gall midges develop in, or on, various organs of many host plant species, where they feed by sucking sap from the tissue. The family Cecidomyiidae have many species that

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# Genetic Variation of the Lesser Peach Tree Borer, *Synanthedon pictipes* (Lepidoptera: Sesiidae) in Arkansas[1]

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**ABSTRACT** The lesser peach tree borer, *Synanthedon pictipes* (LPTB), belongs to the economically important Lepidopteran family Sesiidae. No studies on genetic variation or population structure on the genus *Snyanthedon* have been previously published. We examined DNA sequence variation in a 603 bp region of the mitochondrial cytochrome oxidase I gene (COI), tRNA-leu and cytochrome oxidase II gene (COII) from three LPTB populations in Arkansas. From 114 LPTB collected from three populations, a total of 53 nucleotide positions were polymorphic, and 12 distinct haplotypes were observed. The most frequent haplotype occurred in 88% of the sampled LPTB's and in all three populations. Sequence divergence among haplotypes ranged from 0.2% to 8.8%. According to the standard molecular clock proposed for lepidopteran mtDNA, the haplotypes have been diverging for up to 2.5 million years. The greatest amount of haplotype diversity was observed in the Fayetteville population where borer management is not maintained. High levels of gene flow were observed among the Clarksville, Springdale and Fayetteville populations suggesting the LPTB has a broad dispersal range. Examination of the genealogical relationships and phylogenetic analysis of the 12 haplotypes supports the existence of three genetically distinct but morphologically indistinguishable subspecies.

**KEY WORDS** Genetic variation, mtDNA, Sesiidae, Lepidoptera

*Synanthedon pictipes* (Grote & Robinson) (Lepidoptera: Sesiidae), the lesser peach tree borer (LPTB), is native to North America, and was first reported in Pennsylvania in 1868. LPTB is found east of the Great Plains and north into Canada (Taft et al. 2004). The lesser peach tree borer larvae enter trees at the barks surface where previous injury has occurred (Smith 1951). The larvae feed mostly in the trunk above soil level and within branches (Smith 1951). The larvae will pupate the following spring. The number of generations per year varies by geographic location. More northern states such as New York and South Dakota experience only one generation of LPTB per year (Smith 1951, Gilbertson 1934). Southern states such as Texas, Arkansas, Virginia and Texas have two generations per year (King & Morris 1956, Bobb 1959, Girault 1907, Wong & Cleveland 1968).

Adult emergence of LPTB also varies with geographic location. Emergence is slightly later in more southern locations when compared to the northern locations. Ohio and South Dakota emergence occurs May through September with peak occurrence in June (King 1914, Gilbertson 1934). In Virginia, Georgia,

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Texas, and Arkansas first generation emergence occurs in April through July and second generation emergence occurs early July through November (Bobb 1959, Girault 1907).

The lesser peach tree borer was first reported as a pest of plum and cherry trees. Quaintance (1906) reported the lesser peach tree borer as a pest of importance to peach trees in Georgia. LPTB causes serious damage to peach, cherry, plum, nectarine, and apricot trees. The primary economic impact is caused by girdling of limbs, which is a result of larval feeding. A gradual decline in production on damaged limbs, which when girdled will break under a fruit load and tree loss occurs from repeated girdling (Smith 1951). It is the most important indirect pest of peach and nectarine, with damage resulting in significant loss of production in orchards (Nielson 1978). LPTB feeding can also afford entry for disease organisms, eventually resulting in limb and tree death (Becker 1918). If proper borer management is maintained, sustainability of plantings is drastically increased.

Control of lesser peach tree borers in commercial orchards relies on preventing larval establishment underneath bark of trees. Once under the bark, chemical control is usually ineffective because the larvae are protected. Insecticides should be timed just before or to coincide with egg hatch. To aid in the timing of sprays, pheromone traps are used to monitor for the presence and activity of moths. Insecticidal sprays are applied 7 to 14 d after the first moths are captured in the traps because egg hatch begins about 8 to 10 d after moth emergence. If the delicate unprotected larvae are targeted, LPTB will be adequately controlled. Sprays containing Asana XL, Ambush, Lorsban, Pounce and Thiodan are labeled for control of lesser peachtree borer on peaches.

To determine the most effective time to apply an insecticide, pheromone traps should be used to monitor moth activity. These lures are synthetic copies of the chemicals female moths use to attract their mates. A trap consists of plastic top and bottom held together by a wire hanger with the lure placed inside. The inner surface of the bottom is coated with a sticky material to hold the insects once they land in the trap. Traps are hung in the tree 4 to 5 feet off the ground, usually one for each ten acres of trees in commercial orchards. In order to detect the first activity, traps should be hung in the trees well in advance of the anticipated flight. Proper identification of the moths captured in the trap is essential. There are other clearwing moths that will be attracted to the trap. Captured moths should be examined carefully to be sure that they are the correct species.

Pheromone disruption has been tested for controlling LPTB. Borers are prevented from establishing in the trunks of trees by distributing pheromone ties evenly throughout an orchard, prior to the flight of the LPTB. The ties are attached to the trees at shoulder height. If these are applied at 100 per acre then the male moths are saturated with female pheromone and cannot find the female moths. This results in no fertile eggs and thus no larvae. One application has been shown to provide control for an entire season in northwest Michigan (Thornton 2006, Gary Thornton, District Fruit IPM Agent, Larry Gut, Department of Entomology, Michigan State University). Other methods for preventing LBTB include proper orchard management. Proper pruning techniques should be maintained including thinning fruit to prevent wounds from limb breakage, being careful not to wound trees with harvesting equipment, and preventing sun scald. All wounds on the trees should be cleaned and painted with tree paint.

Dispersal is a key ecological process linking metapopulation dynamics in the landscape to distribution patterns at larger spatial scales. Dispersal can be measured via gene flow. Vandewoeestijne et al. (2004) used this method to determine that the butterfly *Melanargia galathea* had a very low amount of genetic differentiation between populations, which is characteristic of species with a high dispersal capability. No information has been recorded on the dispersal capabilities of any sesiid moth.

Studying the genetic variation among populations also gives insight into the spread of insecticide resistant genes and why populations respond differently to the same control measures. Gene-flow between populations of the cotton bollworm *Helicoverpa armigera* (Hübner) in Australia was evaluated using AMOVA analysis and genetic assignment tests (Scot et al. 2005). The study characterized highly variable patterns of migration of *H. armigera* across cropping regions with some years having high migration between regions, and other years having very little migration. Development of resistance can be exacerbated in periods of low migration because there is no influx of susceptible individuals to dilute the resistance (Scot et al. 2005), but also during periods of high migration because insecticide resistant genes can be transferred rapidly between cropping regions. In years where there is little migration local management practices are of the utmost importance, but when migration increases, national management programs are the keys.

Despite the possible benefits that molecular markers may provide towards diagnostics, dispersal, insecticide resistance, and implementation of area wide control programs, none have been conducted on LPTB or any other sesiid species. The objectives of this study were to determine the extent of genetic variation within and among populations LPTB in Arkansas and to determine the amount of gene flow among populations to determine dispersal capabilities of LPTB.

## Materials and Methods

*Synanthedon pictipes* adults were collected in 2006 using Trécé Pherocon IC wing traps (Trécé Inc., Adair, OK) baited with commercially available pheromone lures from three locations in Arkansas (Table 1): Clarksville from 2 peach orchards that did not practice borer management; Fayetteville from forests bordering an apple orchard where no insecticide pressures were encountered; and Springdale from 2 peach orchards where borer management was practiced. After specimens were collected from traps, they were identified using morphological keys (Eichlin and Duckworth 1988), and stored in glass specimen tubes at −20°C until DNA extraction. Voucher specimens are deposited at the Arthropod Museum, University of Arkansas, Fayetteville, AR.

DNA was extracted from the thoraces of individual LPTB specimens using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 µl of Tris: EDTA and stored at −20°C. Mitochondrial DNA PCR was conducted using primers C1-J-2797 (5′-CCTCGACGTTATTCAGAT-TACC-3′) (Simon et al. 1994) and C2-N-3400 (5′-TCAATATCATTGATGACCAAT-3′) (Taylor et al. 1997). These primers amplify approximately 606 bp of the mtDNA cytochrome oxidase I gene (COI), tRNA-leu and cytochrome oxidase II gene (COII). PCR reactions were conducted using 2 µl of the extracted DNA. The thermal cycler profile for the mtDNA COII gene consisted of 35 cycles of 94°C for

**Table 1. Collection localities (latitude and longitude), sample size (n), and distance between each locality in km.**

| | Springdale, AR n = 31 | Clarksville, AR n = 61 | Fayetteville, AR n = 22 |
|---|---|---|---|
| **Springdale, AR** **36°11′05″N** **94°08′29″W** | 0.00 | | |
| **Clarksville, AR** **35°28′17″N** **94°28′00″W** | 100.6 | 0.00 | |
| **Fayetteville, AR** **36°03′45″N** **94°09′27″W** | 13.7 | 91.2 | 0.00 |

45 s, 46°C for 45 s, and 72°C for 45 s per Szalanski et al. (2000). Amplified DNA from individual sesiids was purified and concentrated using minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega). Samples were sent to the University of Arkansas Medical School Sequencing Facility (Little Rock, AR) for direct sequencing in both directions. Accession numbers for haplotypes submitted to GenBank are EU597680–EU597691.

DNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution (Kimura 1980). Haplotype distribution between populations, number of haplotypes, number of unique haplotypes, haplotype diversity, and average number of pairwise differences were calculated using DNAsp v4.10 (Rozas & Rozas 1999). To test for neutral mutation, the *D* statistics of Tajima (1989) and Fu & Li (1993) was calculated using DNAsp. Genealogical relationships among haplotypes were constructed using TCS (Clement et al. 2000). Tests for differentiation were conducted using AMOVA as implemented in Arlequin v 2.0 (Schneider et al. 2000).

Phylogenetic analysis was conducted using maximum parsimony analysis with the best-fitting evolutionary model as implemented in PAUP* using *Proserpinus clarkiae* (Boisduval) (Lepidoptera: Sphingidae) AF170855, was used as the outgroup taxon. Additional sesiid sequences were obtained from GenBank and from our own sequencing analysis (JAM unpublished data). Bootstrapping was performed using neighbor joining or MP (1000 replicates) to determine the reliability of the obtained topologies.

## Results

DNA sequencing of a portion of the mtDNA COI gene was conducted on 114 individual LPTB sampled from three locations in Arkansas (Table 1). Among the DNA sequences, there were twelve unique haplotypes observed (Table 2). Of the

**Table 2. Haplotype frequencies for 3 locations in Arkansas.**

| Locality | n | Haplotypes (frequency) |
|---|---|---|
| Clarksville, Johnson Co. | 61 | 2, 6, 7, 8, 9, 12 (56) |
| Fayetteville, Washington Co. | 22 | 1, 4 (2), 5 (2), 10, 11, 12 (15) |
| Springdale, Washington Co. | 31 | 3 (2), 12 (29) |

haplotypes discovered, 67% were unique to single specimens. Among the 603 nucleotide sites, 53 nucleotide sites were variable, consisting of 29 nonsynonymous and 24 synonymous mutations (Table 4). The distribution of haplotypes varied among population. Six haplotypes were observed in the Clarksville and Fayetteville populations and two haplotypes in the Springdale population. Haplotype 12 was the most frequent haplotype, occurring in 88% of the sampled LPTB's and in all three populations (Table 2). Genetic divergence ranged from 0.2% (between haplotypes 5 & 4; and 5 & 12) to 8.8% (between haplotypes 2 & 12) (Table 3).

Analysis of molecular variance based upon *S. pictipes* indicated that the within-population heterogeneity, $F_{IS}$ accounted for 93.01% of the molecular variation with respect to haplotypes descent, and was significantly larger than the between-population heterogeneity, $F_{ST}$, which accounted for 6.99% of the molecular variation ($P = 0.003$). Genetic structure was evident between the Clarksville and Fayetteville populations based on Wright's $F_{ST}$ values (Table 5). High levels of gene flow were observed (Nm = 12.56) between the Fayetteville and Springdale populations, which are located 8.48 miles apart (Table 5). The strongly negative values for Fu's F+ and Tajima's D suggest population growth in Clarksville and Fayetteville. Positive D+ and F+ values for Springdale suggests the population is undergoing a reduction in genetic diversity (Table 4).

A TCS spanning tree analysis was conducted on all 12 LPTB haplotypes (Fig. 1). The basal haplotype, 12, was recovered from all three populations. Haplotype 10 and haplotypes 1 and 2 formed distinct clades based on the TCS analysis (Fig. 1) This relationship among the haplotypes was also observed with the maximum parsimony phylogenetic analysis (Fig. 2).

## Discussion

The Clarksville and Fayetteville populations had the greatest amount of genetic variation (Table 2). The Fayetteville population was under no insecticidal pressures and completed life cycles in wild hosts such as cherry and plum trees. The Springdale population was under high insecticidal pressures. Typically, insects under high insecticide pressure undergo a genetic bottleneck.

The LPTB's hosts include a narrow range of plants in the Rosaceae family all in the same genus *Prunus*: peach, cherry, plum, nectarine, and apricot, wild cherry and plum, along with ornamental *Prunus* species (Taft & Snow 2004). Mitter et al. (1978) studied the population genetic consequences of feeding habits of some forest dwelling Lepidoptera and found that specialized feeders have more genetic variation than generalized feeders (feeding on two or more families of host plants). They found that the occupation of two or more host did not lead to substantial genetic substructuring.

**Table 3. Mean (uncorrected) distances (below diagonal) and molecular divergence* (above diagonal) for 12 haplotypes of *Synanthedon pictipes*.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 hap1 | - | 1.44E+05 | 2.16E+06 | 2.09E+06 | 2.16E+06 | 2.31E+06 | 2.16E+06 | 2.16E+06 | 2.31E+06 | 1.30E+06 | 2.52E+06 | 2.09E+06 |
| 2 hap2 | 0.003 | - | 2.31E+06 | 2.24E+06 | 2.31E+06 | 2.45E+06 | 2.31E+06 | 2.31E+06 | 2.45E+06 | 1.44E+06 | 2.67E+06 | 2.24E+06 |
| 3 hap3 | 0.050 | 0.053 | - | 2.17E+05 | 1.44E+05 | 2.88E+05 | 1.44E+05 | 1.44E+05 | 2.88E+05 | 1.59E+06 | 5.05E+05 | 7.22E+04 |
| 4 hap4 | 0.048 | 0.051 | 0.005 | - | 7.22E+04 | 3.60E+05 | 2.17E+05 | 2.17E+05 | 5.65E+08 | 1.66E+06 | 5.77E+05 | 1.44E+05 |
| 5 hap5 | 0.050 | 0.053 | 0.003 | 0.002 | - | 2.88E+05 | 1.44E+05 | 1.44E+05 | 2.88E+05 | 1.59E+06 | 5.05E+05 | 7.22E+04 |
| 6 hap6 | 0.053 | 0.056 | 0.007 | 0.008 | 0.007 | - | 2.88E+05 | 2.88E+05 | 4.33E+05 | 1.73E+06 | 6.49E+05 | 2.17E+05 |
| 7 hap7 | 0.050 | 0.053 | 0.003 | 0.005 | 0.003 | 0.007 | - | 1.44E+05 | 2.88E+05 | 1.59E+06 | 5.05E+05 | 7.22E+04 |
| 8 hap8 | 0.050 | 0.053 | 0.003 | 0.005 | 0.003 | 0.007 | 0.003 | - | 2.88E+05 | 1.59E+06 | 5.05E+05 | 7.22E+04 |
| 9 hap9 | 0.053 | 0.056 | 0.007 | 0.008 | 0.007 | 0.010 | 0.007 | 0.007 | - | 1.73E+06 | 6.49E+05 | 2.17E+05 |
| 10 hap11 | 0.030 | 0.033 | 0.036 | 0.038 | 0.036 | 0.040 | 0.036 | 0.036 | 0.040 | - | 1.95E+06 | 1.51E+06 |
| 11 hap13 | 0.058 | 0.061 | 0.012 | 0.013 | 0.012 | 0.015 | 0.012 | 0.012 | 0.015 | 0.045 | - | 4.33E+05 |
| 12 hap14 | 0.048 | 0.051 | 0.002 | 0.003 | 0.002 | 0.005 | 0.002 | 0.002 | 0.005 | 0.035 | 0.010 | - |

*based on a molecular clock for lepidopteran mtDNA (Brower 1994).

**Table 4. Summary statistics for mtDNA polymorphisms*.**

| Locality | n | NS + S | h | δ (k) | D+ | F+ | D |
|---|---|---|---|---|---|---|---|
| Clarksville | 61 | 20 + 19 | 6 (0.158) | 0.002 (1.28) | −7.06* | −6.54* | −2.8* |
| Fayetteville | 22 | 20 + 22 | 6 (0.537) | 0.009 (5.42) | −1.85 | −2.24 | −2.07* |
| Springdale | 31 | 1 + 0 | 2 (0.125) | 0.0002 (0.125) | 0.591 | 0.245 | −0.77 |
| All | 114 | 29 + 24 | 12 (0.231) | 0.003 (1.78) | −2.65* | −3.15* | −2.58* |

*Note. n = number of sequences; NS + S = number of nonsynonymous and synonymous mutations; h = number of haplotypes (haplotype diversity ± SD shown in parentheses); δ = nucleotide diversity; k = mean number of pairwise nucleotide differences; D+ and F+ statistics are detailed in the text (Fu and Li 1993); D = Tajima's (1989) statistic, detailed in the text. *$P < 0.05$.

Specialists are more likely to develop genetic variation due to local variation in selection coefficients due to lower migration rates in between environmental patches (Mitter 1978). Generalists could have a "homeostatic" mechanism that reduces the environmental variation perceived by loci (Mitter 1978). If specialized species lacked this mechanism chemical changes and differences among host plants could maintain genetic variation that would not be seen in more generalized species. This hypothesis could account for the amount of genetic variation observed among individuals of *S. pictipes*. Many other factors could be influencing genetic variation in this species besides host differences.

A larger amount of genetic variation was found in LPTB compared with other Lepidoptera in both haplotype number and haplotype divergence. Assuming a constant mutation rate of 2.3% per million years Brower (1994), LPTB haplotypes have diverging for approximately 72,200 – 2.52 my (Table 3). Relative to other studies on Lepidoptera genetic variation, this is a high amount of genetic divergence within a species. The haplotype divergence (0.2% to 6.1%) is larger than most variation found among other populations of Lepidoptera. Ohno et al. (2006) found 3 haplotypes of *Ostrinia nubilalis* using mtDNA with divergence 0.15% to 0.73%. Lewter et al. (2006) found that haplotype divergence among 7 fall armyworm (*Spodoptera frugiperda*) haplotypes ranged from 0.164% to 0.329%. The fall armyworm is a more generalized feeder than the LPTB feeding on plants in more than 2 families. *Ostrinia nubilalis* is a very specialized feeder and has very low divergence.

It is not surprising then, given the high amount of genetic divergence observed in LPBT, that there is genetic evidence for three subspecies of LPTB (Figs. 1–2). Figure 1 shows the 95% parsimony network in which haplotypes 1, 2, and 10 are not

**Table 5. Matrix of $F_{ST}$ (above diagonal) and $N_m$ (below diagonal) among three LPTB populations.**

| Locality | 1 | 2 | 3 |
|---|---|---|---|
| 1 Clarksville | — | 0.113 | 0.003 |
| 2 Fayetteville | 43.86 | — | 0.0383 |
| 3 Springdale | 163.15 | 12.56 | — |

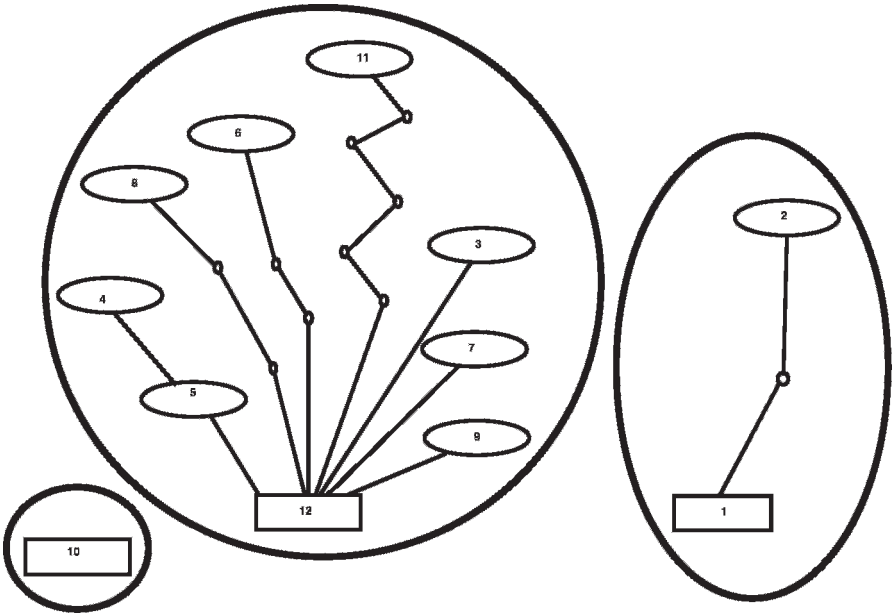


**Fig. 1.** Genealogical relationships among 12 haplotypes of *S. pictipes* estimated by TCS (Clement et al. 2000). A unit branch represents one mutation.

connected into the lineage. Figure 2 depicts a maximum parsimony phylogeny showing the relationship of LPTB relative to other sesiid species. Groups within LPTB formed, with one group containing haplotypes 1, 2, and 10 and the second group containing all other haplotypes. There was strong support for the distinctiveness of haplotypes 12, 1 and 2. No morphological differences were noted between subspecies and no subspecies are noted to exist. Although, 2 subspecies do occur in the closely related *Synanthedon exitiosa* (peach tree borer): *S. exitiosa graefi* and *S. exitiosa edwardsii* (Eichlin & Duckworth 1988).

Since all subspecies were collected with the same pheromone, it is unlikely that pheromone races exist in *S. pictipes*, but not altogether improbable. In Lepidoptera, pheromone races are not uncommon. Felix et al. (1996) found mtDNA variation among pheromone races of the dingy cutworm, *Feltia jaculifera* (Gn.). Studies on the genetic variation of sesiid species should be expanded. More species should be investigated especially those of economic importance. This is the first study of genetic variation among populations of a sesiid moth.

High levels of genetic variation were observed among haplotypes of LPTB in this study, however, 88% of the sampled moths belonged to a single haplotype. This gives genetic evidence that there is considerable movement of a single haplotype among populations, which is supported by the large Nm value observed.

Despite the high levels of gene flow, there is considerable evidence partitioning of haplotypes among populations. For example, Wright's FST values do show some genetic structure among populations (when values of FST are above 0.05) and this value was the largest between the Fayetteville and Clarksville populations. Also, while there was one haplotype that was very

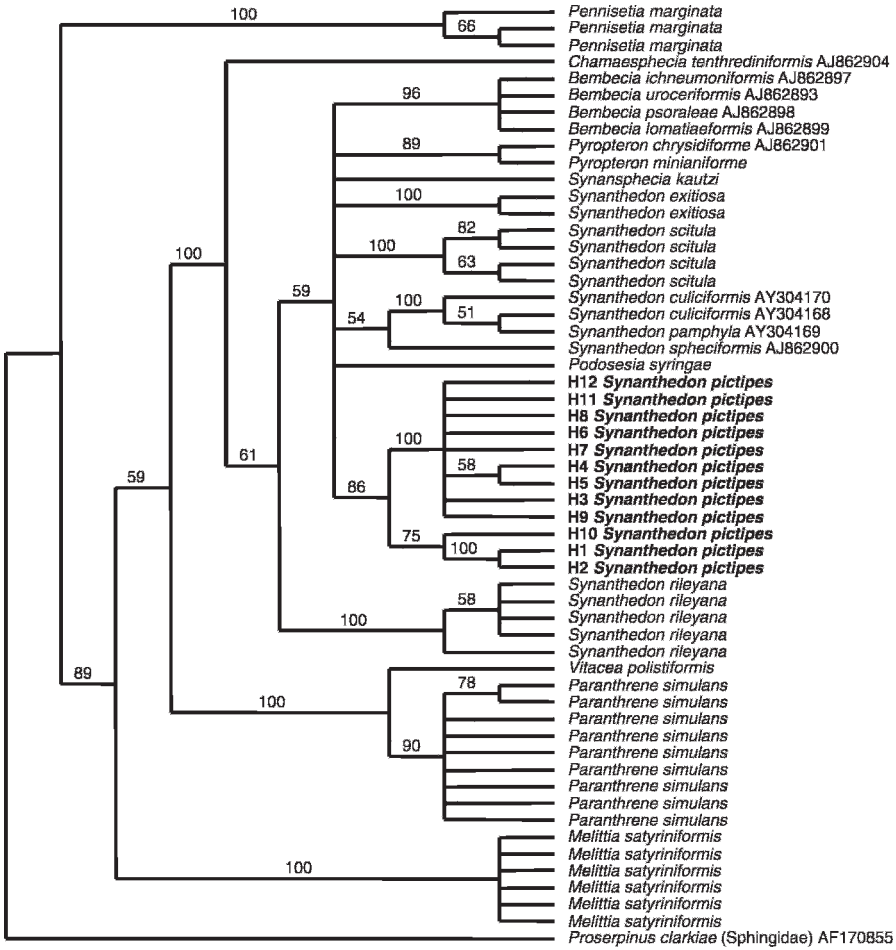


**Fig. 2.** Phylogenetic relationships among 12 haplotypes of *S. picitpes* relative to 19 other species of Sesiidae calculated by MP in Paup* 4.0b10 (Swofford 2001).

frequent, the majority of the haplotypes was singleton (occurring only once) and was not found among populations. This in combination with the TCS genealogical spanning tree's support for three distinct lineages, and the large amount of genetic divergence among haplotypes gives considerable genetic support for multiple morphologically identical subspecies of LPTB. Based on these observations, it is evident that considerable genetic research remains to be conducted on sesiids in order to obtain a better understanding of the evolutionary relationship among populations.

Future studies should include the use of nuclear markers to confirm the existence of subspecies, a broader sampling methodology and the use of modified pheromone traps to determine if there are pheromone races in the lesser peach tree borer.

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# Genetic Variation of the Lesser Peach Tree Borer, *Synanthedon pictipes* (Lepidoptera: Sesiidae) in Arkansas[1]

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J. Agric. Urban Entomol. 25(1): 25–35 (January 2008)

**ABSTRACT** The lesser peach tree borer, *Synanthedon pictipes* (LPTB), belongs to the economically important Lepidopteran family Sesiidae. No studies on genetic variation or population structure on the genus *Snyanthedon* have been previously published. We examined DNA sequence variation in a 603 bp region of the mitochondrial cytochrome oxidase I gene (COI), tRNA-leu and cytochrome oxidase II gene (COII) from three LPTB populations in Arkansas. From 114 LPTB collected from three populations, a total of 53 nucleotide positions were polymorphic, and 12 distinct haplotypes were observed. The most frequent haplotype occurred in 88% of the sampled LPTB's and in all three populations. Sequence divergence among haplotypes ranged from 0.2% to 8.8%. According to the standard molecular clock proposed for lepidopteran mtDNA, the haplotypes have been diverging for up to 2.5 million years. The greatest amount of haplotype diversity was observed in the Fayetteville population where borer management is not maintained. High levels of gene flow were observed among the Clarksville, Springdale and Fayetteville populations suggesting the LPTB has a broad dispersal range. Examination of the genealogical relationships and phylogenetic analysis of the 12 haplotypes supports the existence of three genetically distinct but morphologically indistinguishable subspecies.

**KEY WORDS** Genetic variation, mtDNA, Sesiidae, Lepidoptera

*Synanthedon pictipes* (Grote & Robinson) (Lepidoptera: Sesiidae), the lesser peach tree borer (LPTB), is native to North America, and was first reported in Pennsylvania in 1868. LPTB is found east of the Great Plains and north into Canada (Taft et al. 2004). The lesser peach tree borer larvae enter trees at the barks surface where previous injury has occurred (Smith 1951). The larvae feed mostly in the trunk above soil level and within branches (Smith 1951). The larvae will pupate the following spring. The number of generations per year varies by geographic location. More northern states such as New York and South Dakota experience only one generation of LPTB per year (Smith 1951, Gilbertson 1934). Southern states such as Texas, Arkansas, Virginia and Texas have two generations per year (King & Morris 1956, Bobb 1959, Girault 1907, Wong & Cleveland 1968).

Adult emergence of LPTB also varies with geographic location. Emergence is slightly later in more southern locations when compared to the northern locations. Ohio and South Dakota emergence occurs May through September with peak occurrence in June (King 1914, Gilbertson 1934). In Virginia, Georgia,

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# First Report of the Papaya Mealybug, *Paracoccus marginatus* (Hemiptera: Pseudococcidae), in Indonesia and India[1]

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**ABSTRACT** The papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae), is recorded from the Oriental Region for the first time, where it was found in Indonesia (Java) and India (Tamil Nadu) in 2008. Papaya mealybug is a polyphagous pest that damages many tropical crops. A native of Central America, it spread to the Caribbean region and South America in the 1990s; since then it has been accidentally introduced to some islands in the Pacific region. The distribution, host range and characteristics of the mealybug are summarized.

**KEY WORDS** *Paracoccus marginatus*, mealybug, pest, Indonesia, India, papaya

The papaya mealybug (PM), *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) is native to Central America (Mexico, Belize, Guatemala and Costa Rica). The mealybug is a polyphagous pest, with hosts recorded from 22 plant families including economic and weedy plants in genera such as *Acacia*, *Acalypha*, *Ananas*, *Annona*, *Bidens*, *Capsicum*, *Hibiscus*, *Ipomoea*, *Mangifera*, *Manihot*, *Persea*, *Plumeria*, *Punica*, *Solanum* and *Vigna* (Ben-Dov 2008). *Carica papaya* (L.) H. Karst. and *Parthenium hysterophorus* L. are favored hosts. Sooty mold developing on honeydew excreted by this mealybug covers the leaves, fruits and stems, impeding photosynthesis and gaseous exchange. Damage symptoms caused by PM resemble those induced by *Maconellicoccus hirsutus* (Green), the pink hibiscus mealybug. On papaya, PM infests the veins of older leaves, which turn yellow, dry up and are shed prematurely, and all parts of young leaves and fruits. Tender leaves become crinkled and curly; flowers and young fruits drop and shoots become bunchy.

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Papaya trees die within a few months of becoming infested. PM infestation of *Plumeria* causes the leaves to curl and new leaves fail to expand fully. On *Hibiscus*, leaves and flowers attacked by PM become distorted and the shoots appear bunchy.

*Paracoccus marginatus* reproduces sexually. The live adult female (about 2.5 mm long and 1.5 mm wide) is evenly covered in powdery, white wax, without any longitudinal depressions. Short waxy filaments develop around the body margin including short caudal filaments. The body contents are yellow in life but turn black in less than one day after death, even when preserved in alcohol. In slide-mounted adult females from the Oriental region, this is the only species of *Paracoccus* that totally lacks oral rim ducts in the sub-median or median areas of the dorsum. There are three nymphal instars and no pupal stage in the wingless female, and eggs are laid in a small, white ovisac of woolly wax. The winged male has two nymphal stages, a pre-pupa and a pupal stage. There are several generations per year.

Although specimens of PM were first collected in Mexico in 1955, the species was only described in 1992 by Williams and Granara de Willink (1992). Since then, it has spread outside its native region (Miller et al. 1999), being recorded from the U.S. Virgin Islands, Dominican Republic and Grenada in 1994; Antigua, Saint Martin and British Virgin Islands in 1996; U.S.A. (Florida), Haiti, St. Kitts and Nevis, St. Barthélemy and Guadaloupe in 1998; French Guyana, Cuba and Puerto Rico in 1999; Barbados, Cayman Islands and Montserrat in 2000; the Bahamas and Guam in 2002; Palau in 2003; Hawaii (Maui and Oahu in 2004, Hawaii in 2006); and the Northern Marianas (Tinian) in 2005 (Matile-Ferrero & Étienne 1998, Halbert 1998, Pollard 1999, Watson & Chandler 2000, Martinez et al. 2000, Matile Ferrero et al. 2000, CABI/EPPO 2000, Meyerdirk et al. 2004, Muniappan et al. 2006, Heu et al. 2007). In May 2008, scientists from the Integrated Pest Management Collaborative Research Support Program (IPM CRSP) supported by U.S.A.I.D. were carrying out surveys in Java, Indonesia, when they found a papaya tree infested with PM in Bogor Botanical Garden. Subsequent surveys revealed that thousands of papaya trees were infested by PM. In July 2008, another team of scientists from the same organization visiting Coimbatore, India found PM infesting papaya in the orchard at Tamil Nadu Agricultural University.

The recent establishment of PM in West Java, Indonesia and Tamil Nadu, India is of serious concern because these are the first reports of its occurrence in the Oriental region. Within three months of the first discovery of *P. marginatus* in Indonesia, the magnitude of the mealybug problem has caused the government to consider development of a classical biological control program against it (A. Rauf, personal communication). Similar efforts are being made to initiate a classical biological control program in India to prevent the pest from spreading and causing severe damage. Papaya mealybug will certainly spread to rest of Indonesia, India and other countries in the Oriental region, and could cause losses worth millions of dollars in crops such as papaya, cassava, sour sop, sweet sop, mango, avocado, and ornamental plants such as *Hibiscus* and *Plumeria*.

*Paracoccus marginatus* is an exotic introduced pest, so it is suitable for a classical biological control approach. This method has been successfully implemented against PM in several countries in the Caribbean, some islands in the Pacific and in the states of Florida and Hawaii in the United States

(Meyerdirk et al. 2004, Muniappan et al. 2006, Heu et al. 2007). In 1999, USDA-ARS scientists and cooperators collected the parasitoids *Anagyrus loecki* Noyes, *Acerophagous papayae* Noyes and Schauff and *Pseudleptomastrix mexicana* Noyes and Schauff (Hymenoptera: Encyrtidae) from PM in Mexico (Noyes & Schauff 2003). All three species were screened for hyperparasites at the USDA-ARS Beneficial Insects Laboratory in Newark, Delaware, U.S.A. Environmental Assessments were prepared by Meyerdirk (1999, 2000) and the parasitoids were shipped to San Juan, Puerto Rico, where they are being mass produced in a cooperative effort with the Puerto Rico Department of Agriculture and USDA-APHIS PPQ. These parasitoids have been sent to most of the countries that PM has invaded in recent years, and have successfully solved the pest problem in each case. To prevent devastating losses to several crops in Indonesia and India, a classical biological control program against *P. marginatus* should be implemented right away. Early control of the pest is important also to reduce the likelihood of spread of PM to other countries in the region.

## Acknowledgments

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# First Report of the Papaya Mealybug, *Paracoccus marginatus* (Hemiptera: Pseudococcidae), in Indonesia and India[1]

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J. Agric. Urban Entomol. 25(1): 37–40 (January 2008)

**ABSTRACT** The papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae), is recorded from the Oriental Region for the first time, where it was found in Indonesia (Java) and India (Tamil Nadu) in 2008. Papaya mealybug is a polyphagous pest that damages many tropical crops. A native of Central America, it spread to the Caribbean region and South America in the 1990s; since then it has been accidentally introduced to some islands in the Pacific region. The distribution, host range and characteristics of the mealybug are summarized.

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# Susceptibility of the Bed Bug *Cimex lectularius* L. (Heteroptera: Cimicidae) Collected in Poultry Production Facilities to Selected Insecticides[1]

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**ABSTRACT** *Cimex lectularius* L. is a widespread hematophagus insect pest around the world and is currently experiencing a reemergence as a public health pest of concern. One possible source of bed bugs to the human environment is the movement of bed bugs from poultry facilities to human structures by poultry workers. No recent studies have been conducted on the susceptibility of this insect to a wide range of insecticides. In addition, populations of bed bugs from poultry facilities have not been screened against insecticides for over 15 yr. Adult bed bugs collected from three poultry facilities in northwest Arkansas were exposed for 24 or 48 h (25°C) to glass vials treated with various dilutions of 12 insecticides dissolved in acetone to determine the concentration–response relationship. The order of toxicity, from most to least based on the $LC_{50}$s was: λ-cyhalothrin, bifenthrin, carbaryl, imidacloprid, fipronil, permethrin, diazinon, spinosyn, dichlorvos, chlorfenapyr, and DDT. Significant differences in $LC_{50}$ and $LC_{90}$ values for diazinon was observed among the three populations due to the previous history of repeated exposure to a mixture of tetrachlorvinphos and dichlorvos over a 10 yr period when compared to the $LC_{50}$s of two populations that had been exposed to the tetrachlorvinphos and dichlorvos mixture during 2–3 flock cycles. Bed bugs in each of the three populations exhibited high levels of DDT resistance, $LC_{50} >$ 100,000 ppm, which confirms that resistance to this insecticide continues in bed bug populations. This study documents baseline toxicological data for 12 insecticides in three populations of bed bugs and provides the first data on bed bug susceptibility to fipronil, spinosyn, and imidacloprid.

**KEY WORDS** bed bug, *Cimex lectularius*, insecticide resistance

*Cimex lectularius* L. (Heteroptera: Cimicidae) is a hematophagous insect that can be a major pest in breeder poultry facilities (Axtell 1985) and has regained worldwide attention due to its recent resurgence into dwellings shared by humans. Both sexes feed on blood and require a blood meal for subsequent molts (Usinger 1966). Although active dispersal of bed bugs can be important, passive dispersal is almost exclusively their dispersal *modus operandi*. This species is easily translocated by passive dispersal and adapts to multiple hosts (Usinger 1966, Marshall 1981, Lehane 2005). Consequently, when host animals including

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humans are unavailable, it is extremely difficult to isolate the origins of recent infestations. However, basic molecular biology tools can be used to elucidate the identity of hosts fed upon by bed bugs, hence providing the potential for forensic applications into host identity from undigested blood obtained from bed bugs (Szalanski et al. 2006).

Passive dispersal is the most important way for the wingless cimicids to reach new hosts. Increasing numbers of humans are moving or being moved across international boundaries in many parts of the world. Bed bugs can be transported by humans in clothing and luggage (Axtell 1999, Boase 2001, Krinsky 2002), and have been detected on people traveling by airplanes, trains, ships, and cars. In poultry production, bed bugs are transported from infested facilities by human shoes, clothes, egg boxes and production equipment (Steelman 2000). Population genetic analysis of bed bugs revealed that mitochondrial 16S rRNA haplotypes are shared between poultry facilities and human structures (Szalanski et al. 2008). In addition, wild and domesticated birds and bats are often hosts of *C. lectularius* and are important in their dispersal. Since the late 1970s, bed bugs have undergone a resurgence, became widespread in the late 1990s, and appears to be cosmopolitan across the developed world (Reinhardt & Siva-Jothy 2007). Reasons for this resurgence may include increased long range air travel (Boase 2001), the ability of bed bugs to disperse locally, reduction in the use of residual insecticides around structures, and movement of bed bugs from birds and bats to domesticated birds and then to humans. Bed bugs are also an important pest of poultry (Axtel and Arends 1990, Usinger 1966, Steelman 2000, Mullen & Durden 2002). Each side of a broiler-breeder house typically has a wooden slatted platform over which the feeders and waters are hung. These wooden slats provide an ideal environment for bed bugs (Fletcher & Axtell 1993). One of the most plausible explanations for bed bug resurgence lies in this group's adaptive ability to alternate hosts (Meyers 1928, Kemper 1936, Went 1939, 1941, Hase 1964, Overal & Wingate 1976, Stelmaszyk 1986) and phoretically translocate with workers in poultry facilities (Jacobs 2005) where they can amass in large numbers (Lyon 1995, Steelman 2000).

Insecticide resistance in bed bugs was first observed with the organochlorine DDT (Lofgren 1958, Busvine 1958), and later to the organophosphates malathion and diazinon (Feroz 1968). Recently, populations of *C. lectularius* collected from human structures in the United States have been shown to have high levels of pyrethroid resistance (Moore & Miller 2006, Romero et al. 2007) and has been previously attributed as a consequence of DDT cross-resistance (Busvine 1958).

This report compares the relative susceptibility of three populations of bed bugs collected from broiler-breeder egg production facilities to 12 insecticidal compounds.

## Materials and Methods

The bed bugs used in this study were field collected from Arkansas broiler-breeder poultry facilities located in Washington (Population 1, northwest Arkansas), Carroll (Population 2, northwest Arkansas) and Lafayette (Population 3, southwest Arkansas) counties during July 2007. Broiler-breeder egg production flock cycles have approximate 45 week duration after which time all chickens are removed and generally a 4 week period elapses before the next flock

is placed in the facility. After each flock cycle at the poultry farm where Population 1 was collected the facility was treated with pyrethroids (permethrin and a permethrin-λ cyhalothrin mix), tetrachlorvinphos and dichlorvos (organophosphates), and spinosyn.

The poultry production facilities where Population 2 was collected had been treated between production flocks as well as during the flock cycle with the organophosphate insecticide combination RaVap® (tetrachlorvinphos and dichlorvos) for approximately 10 yr in consistent attempts to manage the bed bug population. The poultry farm where Population 3 was collected had been treated between flock cycles with cyhalothrin and tetrachlorvinphos and dichlorvos combined (RaVap®) when chickens were present in the facility for 3 yr.

The bed bug adults used in the insecticide susceptibility tests were collected from beneath the egg pad in the nest boxes, along the area where the slatted flooring joined the walls and from the wooden wall studs. A soft bristle brush was used to sweep the adult bugs into a plastic dust pan. The bugs were placed in 30 × 25 × 8 cm plastic storage containers with lids for transportation to the Veterinary Entomology laboratory, University of Arkansas, Fayetteville, AR. In the lab, adult bed bugs were removed from the storage containers using forceps and placed in 150 × 25 mm plastic Petri dishes lined with filter paper. Bed bugs were maintained in the Petri dishes at 25°C. Samples of the adult bed bugs were morphologically identified using descriptions outlined by Usinger (1966) and molecular diagnostics using mitochondrial 16S rRNA sequencing per Szalanski et al. (2008). Voucher specimens were deposited in the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR, USA.

The adult vial test (AVT) used in this study was originally developed by Plapp and Plapp (1987) for adult tobacco budworm, *Heliothis virescens* (F.), and it has since been modified for several other insect species (Cilek et al. 1991, Snodgrass 1996, Amalin et al. 2000). This insecticide-coated glass was used to determine the susceptibility of adult bed bugs to 12 pesticides: permethrin, λ-cyhalothrin, bifenthrin, diazinon, dichlorvos, tetrachlorvinphos (Rabon®), carbaryl, DDT, spinosyn (Extinosad®), imidacloprid (Provado®), chlorfenapyr, and fipronil. The spinosyn (Extinosad®) utilized was commercial grade, while the permethrin, λ-cyhalothrin, bifenthrin, diazinon, dichlorvos, tetrachlorvinphos (Rabon®), carbaryl, DDT, imidacloprid, chlorfenapyr, and fipronil were purchased as the technical grade from Chem Service (West Chester, PA). The 12 insecticides represents eight insecticide classes including pyrethroids, organophosphates, carbamates, organochlorines, spinosyn, chloronicotinyl, pyrrole, and phenylpyrazole (Table 1).

All pesticides were dissolved in acetone and serially diluted. Treated vials were hand rotated until all surfaces within vials had been coated and the acetone had completely evaporated, leaving a uniformly applied insecticidal residue on the inner surface. The number and range of concentrations varied for each insecticide tested. Insecticide, number of concentrations, and range of concentrations in ppm per vial, respectively, were: permethrin 5, 5–500; λ-cyhalothrin 5, 0.05–500; bifenthrin, 4, 0.5–500; diazinon, 4, 5–1000; dichlorvos, 3, 5–500; tetrachlorvinphos, 3, 5–500; carbaryl, 3, 5–500; DDT, 5, 5–100,000; spinosyn, 3, 5–500; imidacloprid, 4, 0.5–500; chlorfenapyr, 4, 5–1000; and fipronil, 4, 0.5–50. Vials treated with only acetone were used as controls. For each treatment 10 adult blood-fed bed bugs were used. Each treatment of 10 bed bugs was replicated

**Table 1. Concentration – response *of Cimex lectularius*, exposed for 24 or 48 h (25°C) to insecticide treated vials. $LC_{50}$ and $LC_{90}$ in ppm with 95% confidence intervals based on log-probit analysis. Population 1 (Brentwood, AR), Population 2 (Berryville, AR 2*), population 3 (Gin City, AR).**

| Insecticide | Pop | h | n | $LC_{50}$ (95% CL) | $LC_{90}$ (95% CL) | Slope ± SE | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Permethrin[a] | 1 | 24 | 150 | 8.2 (15.4–21.1)[A] | 27.6 (23.9–34.5) | 0.23 ± 0.04 | 30.73 |
| Permethrin[a] | 2 | 24 | 150 | 37.1 (32.3–42.8)[A] | 53.7 (47.1–65.0) | 0.13 ± 0.02 | 38.05 |
| Permethrin[a] | 3 | 24 | 150 | 22.5 (14.9–32.5)[A] | 52.3 (40.2–76.5) | 0.07 ± 0.01 | 27.92 |
| λ-cyhalothrin[a] | 1 | 24 | 150 | 1.5 (0.9–2.7)[A] | 3.4 (2.3–6.5) | 1.15 ± 0.30 | 14.28 |
| λ-cyhalothrin[a] | 2 | 24 | 150 | 5.0 (4.2–6.2)[A] | 7.5 (6.3–11.0) | 0.88 ± 0.23 | 14.18 |
| λ-cyhalothrin[a] | 3 | 24 | 150 | 0.7 (0.3–1.8)[A] | 4.3 (2.9–8.5) | 0.62 ± .018 | 11.48 |
| Bifenthrin[a] | 1 | 24 | 150 | 21.7 (14.1–30.3)[A] | 48.7 (38.5–67.0) | 0.08 ± 0.01 | 33.82 |
| Bifenthrin[a] | 2 | 24 | 150 | 2.3 (1.4–3.4)[A] | 4.1 (3.0–6.1) | 1.24 ± 0.26 | 22.04 |
| Bifenthrin[a] | 3 | 24 | 150 | 6.5 (4.6–22.8)[A] | 12.5 (8.3–62.6) | 0.37 ± 0.16 | 5.30 |
| Diazinon[b] | 1 | 24 | 150 | 28.3 (24.9–34.1)[A] | 39.3 (33.7–53.8) | 5.66 ± 1.24 | 20.86 |
| Diazinon[b] | 2 | 24 | 150 | 561.5 (457.3–792.1)[D] | 900.5 (708.9–1490.9) | 3.64 ± 0.79 | 13.40 |
| Diazinon[b] | 3 | 24 | 150 | 9.8 (0.4–19.5)[A] | 36.0 (24.6–71.3) | 0.08 ± 0.02 | 12.13 |
| Dichlorvos[b] | 1 | 24 | 150 | 750.1 (635.1–865.4)[D] | 913.1 (805.8–1089.1) | 0.01 ± .008 | 22.11 |
| Dichlorvos[b] | 2 | 24 | 150 | 170.9 (110.1–291.0)[BC] | 358.6 (253.6–627.7) | 2.00 ± 0.39 | 17.61 |
| Dichlorvos[b] | 3 | 24 | 150 | 239.9 (148.8–360.0)[C] | 643.9 (483.9–1011.2) | 0.01 ± 0.01 | 21.23 |
| Carbaryl[c] | 1 | 24 | 145 | 27.7 (20.0–36.8)[A] | 45.9 (36.7–61.0) | 0.12 ± 0.02 | 34.55 |
| Carbaryl[c] | 2 | 24 | 150 | 3.4 (2.3–4.2)[A] | 5.3 (4.5–6.7) | 1.16 ± 0.26 | 20.75 |
| Carbaryl[c] | 3 | 24 | 150 | 5.0 (3.2–14.4)[A] | 12.1 (9.9–54.5) | 0.31 ± 0.13 | 5.63 |
| DDT[d] | 1 | 24 | 210 | >100,000 | | | |
| DDT[d] | 2 | 24 | 90 | >100,000 | | | |
| DDT[d] | 3 | 24 | 70 | >100,000 | | | |
| Spinosyn[e] | 1 | 48 | 150 | 69.3 (52.8–344.2)[B] | 111.3 (77.3–825.7) | 3.63 ± 1.13 | 10.30 |
| Spinosyn[e] | 2 | 48 | 150 | 169.3 (111.1–293.4)[B] | 357.3 (255.6–629.4) | 2.01 ± 0.38 | 18.63 |
| Spinosyn[e] | 3 | 48 | 150 | 650.9 (459.1–1427.8)[D] | 1269.6 (859.5–3285.2) | 2.31 ± 0.50 | 8.50 |
| Imidacloprid[f] | 1 | 24 | 150 | 6.17 (4.9–13.5)[A] | 9.9 (7.3–31.5) | 0.59 ± 0.24 | 6.04 |

**Table 1. Continued.**

| Insecticide | Pop | h | n | $LC_{50}$ (95% CL) | $LC_{90}$ (95% CL) | Slope ± SE | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Imidacloprid[f] | 2 | 24 | 150 | 3.8 (2.6–5.2)[A] | 7.6 (6.0–12.2) | 0.57 ± 0.15 | 15.00 |
| Imidacloprid[f] | 3 | 24 | 150 | 0.15 (−5.5–1.69)[A] | 5.8 (3.8–17.0) | 0.39 ± 0.14 | 7.15 |
| Chlorfenapyr[g] | 1 | 48 | 150 | 617.4 (489.6–987.5)[D] | 996.5 (754.5–1899.4) | 0.01 ± .001 | 10.80 |
| Chlorfenapyr[g] | 2 | 48 | 150 | 517.6 (425.2–680.0)[D] | 832.3 (672.7–1260.7) | 0.01 ± .001 | 16.00 |
| Chlorfenapyr[g] | 3 | 48 | 150 | 104.6 (53.6–218.6)[B] | 310.1 (203.8–696.1) | 0.01 ± .003 | 11.12 |
| Fipronil[h] | 1 | 48 | 150 | 7.8 (1.2–16.3)[A] | 34.7 (23.4–70.5) | 0.64 ± 0.29 | 4.95 |
| Fipronil[h] | 2 | 48 | 150 | 34.8 (26.7–42.7)[A] | 55.3 (46.9–69.2) | 0.11 ± 0.02 | 32.23 |
| Fipronil[h] | 3 | 48 | 150 | 30.1 (20.8–38.9)[A] | 61.2 (51.4–79.8) | 0.07 ± 0.01 | 25.67 |

[a]pyrethroid.
[b]organophosphate.
[c]carbamate.
[d]organochlorine.
[e]spinosyn.
[f]chloronicotinyl.
[g]pyrrole.
[h]phenylpyrazole.
$LC_{50}$ data within rows or columns followed by different capital letters are significantly different at $P < 0.05$.

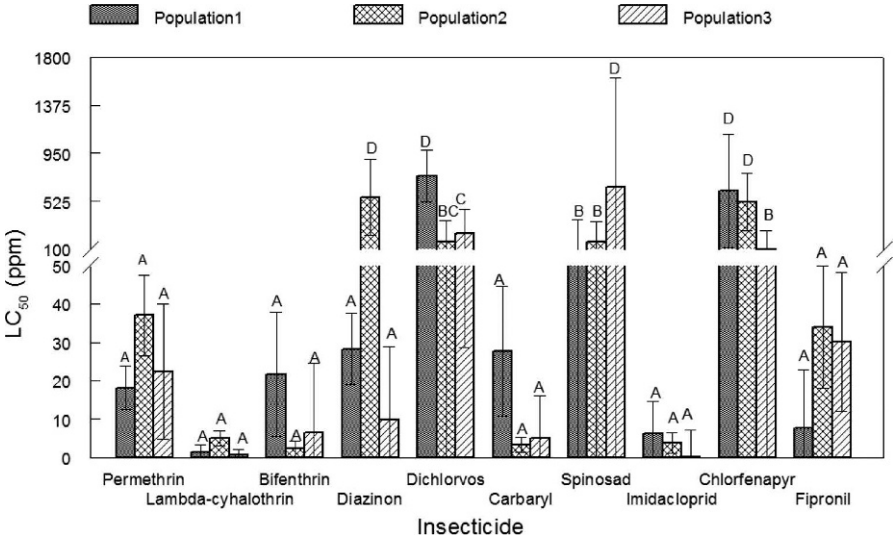


**Fig. 1.** $LC_{50}$ (±C.L.) response of three bed bug populations to selected insecticides.

3 times for each concentration. Vials were placed upright in a ventilated cabinet within a fume hood and maintained at a constant temperature of 25°C and 80% RH for 24 or 48 h.

Mortality of bed bugs was determined immediately after the 24-h period. A bed bug was considered dead if it was not moving or could not right itself when probed. Percentage mortality was measured as the proportion of 30 bed bugs dead after a 24-h exposure to the pesticides. All data were subjected to probit analysis (PROC PROBIT, SAS Institute 2004).

## Results and Discussion

Susceptibility of bed bugs varied among insecticides and populations collected for testing (Table 1). The test for lack-of-fit of the probit model was not significant in all cases (Pearson chi-square test; $P > 0.05$). No control mortality occurred in this study. Because we conducted probit analysis separately on each bed bug population we could not use any kind of design structure. The probit analysis generated one $LC_{50}$ for each insecticide/population, thus, the lack of $LC_{50}$ replication prevented the use of an ANOVA for population evaluation. However, the probit analysis generated a Confidence Interval for each $LC_{50}$ and that allowed us to compare the populations overlaps of the Confidence Intervals. These comparisons were used along with the historical facility treatment information to explain differences in susceptibility response among the three bed bug populations.

Bed bugs from the three poultry farms were all susceptible to the pyrethroids with $LC_{50}$ values ranging from 0.7 to 37.1 ppm for bifenthrin, λ-cyhalothrin, and permethrin (Table 1 and Fig. 1). In addition, $LC_{50}$ comparisons indicated that there was no significant difference in the susceptibility responses among the

three bed bug populations to the same synthetic pyrethroid. Historically, Population 1, had been exposed to one spray application of permethrin in 2005, and one spray application of cyhalothrin and permethrin mixed in 2006, with all applications occurring during flock intervals when no chickens were present in the facilities. Population 2 had received no exposure to any pyrethroid while Population 3 had been exposed to facility treatment with cyhalothrin dust between flocks in 2006 and 2007.

Two representative organophosphate insecticides (diazinon and dichlorvos) were used in our laboratory tests of susceptibility. Comparison of confidence limits (CL) indicated that Population 2 was resistant to diazinon and dichlorvos (Fig. 1). No significant difference existed between the $LC_{50}$s of Population 1 and 3 while both were significantly more susceptible than Population 2. The facility containing Population 2 had been repeatedly treated with RaVap® (a combination of tetrachlorvinphos and dichlorvos) over several years while chickens were in the facility and between flock production cycles. Population 1 was treated three times during 2006 and one time in 2007 with tetrachlorvinphos during the flock production cycles and sprayed with RaVap® two times during each production cycle. All three populations were significantly less susceptible to dichlorvos than to any of the pyrethroid insecticides tested (Fig. 1). Bed bugs from Population 1 was significantly more resistant to dichlorvos than the bed bugs from Population 3 while no significant difference was found between the susceptibility of bed bugs from Population 2 and 3 (Table 1, Fig. 1).

Carbaryl was used in the laboratory tests to represent the carbamate family of insecticides and no significant difference in susceptibility existed among the three populations of bed bugs (Table 1). Historically, no carbaryl or other carbamate had been used in the broiler-breeder facilities. No significant difference existed between the $LC_{50}$s of the three populations and the $LC_{50}$s found for the pyrethroids tested on these bed bugs. This is a significant finding since pyrethroids have been implicated as having poor residual control on bed bugs in urban application scenarios (Moore & Miller 2006, Romero et al. 2006).

No previous history of organochlorine use was obtained for any of the facilities containing the three populations of bed bugs utilized in the present studies as all three facilities were constructed after the ban on organochlorine insecticides in the United States over 30 yr ago. However, we included DDT in these studies and found that the $LC_{50}$s of all three populations were >100,000 ppm. It seems probable that all three of these bed bug populations had been exposed to DDT or other organochlorines at some previous time, and certainly before the infestations occurred in the broiler-breeder egg production facilities. High levels of resistance to DDT, effectively acting as 99% exposure, caused only 20% mortality after 96 h of continuous exposure. Bed bugs have had a history of resistance to DDT for over 50 yr. In the 1950s, resistance to DDT was observed in bed bugs from Japan, Korea, Ohio, and U.S. naval vessels (Lofgren et al. 1958). Populations of *C. lectularius* from South Korea had a DDT $LC_{50}$ of 2.8% and a dieldrin $LC_{50}$ of 0.167% (Cha et al. 1970). More recently, bed bugs from Brazil have been found to have a $LD_{50}$ greater than 4% DDT (Nagem & Williams 1992), and high levels of DDT resistance has also been observed in *Cimex hemipterus* (F) from Sri Lanka with 41–88% survival to 2% DDT treated paper (Karunaratne et al. 2006). *C. hemipterus* has also been found to have resistance to the pyrethroids, permethrin and alpha-cypermethrin, from Tanzania (Myamba et al. 2002).

Although cross-resistance from DDT to pyrethroids is believed to exacerbate the ongoing reemergence of this pest, it has not been empirically proven. Resistance has been defined as, "the development of a strain capable of surviving a dose lethal to a majority of individuals in a normal population" (ffrench-Constant & Roush 1990). However, what defines a "normal" population? It is apparent from these field collected populations that the only consistent relationship in the quantal response to insecticides is that a significant population bottleneck constrained most populations from the widespread application of DDT, thus skewing their genetic constitutions. Robertson & Preisler (1992) defines resistance as a significant, genetically based shift in the molecular, biochemical, or behavioral bases of quantal responses in populations of an arthropod species, whereby resistance represents one extreme of response, compared with susceptibility, the other extreme…various degrees of tolerance lie between the two extremes.

New and novel insecticides were also tested to determine the base line susceptibility of bed bugs infesting poultry production facilities. Imidacloprid (a chlorinated analog of nicotine) caused $LC_{50}$s in all three bed bug populations that were not significantly different and were not significantly different from the pyrethroid $LC_{50}$s (Table 1, Fig. 1). This product had not been used for bed bug control in any of the broiler-breeder egg production facilities.

The $LC_{50}$ data for a formulation of spinosyn (a mixture of spinosyn A and D), showed that the bed bugs from the three populations responded much slower to this compound than to the pyrethroid insecticides (Fig. 1). Only the facilities containing Population 1 had previously been treated with a formulation of spinosad; however, no significant difference was found in the $LC_{50}$ data for Population 1 and Population 2. Both populations were significantly more susceptible than the bed bugs comprising Population 3 (Fig. 1). In addition, the $LC_{50}$ was reached 48 h after exposure rather than the 24 h obtained for the pyrethroids and organophosphate compounds tested. Chlorfenapyr (halogenated pyrrole) another microbiologically produced compound caused $LC_{50}$s to the three populations of bed buds similar to the data obtained for spinosyn. Chlorfenapyr had not been used at any of the three poultry farms. This observation was consistent with results from Miller & Moore (2006), wherein they reported mortality to chlorfenapyr bioassays resulted in $LT_{50}$ (lethal time) of 48 h after bed bug exposure. Fipronil (a phenylpyrazole) caused significantly lower $LC_{50}$s to the bed bugs in all three populations than did either spinosyn or chlorfenapyr but were not significantly lower than the $LC_{50}$s obtained for the pyrethroids. However, as was observed for spinosyn and chlorfenapyr, the $LC_{50}$s for the three populations were not reached until 48 h after exposure.

As evidenced by the relative susceptibility of bed bugs to pyrethroids in this study, it would be imprudent to suggest that pyrethroids are ineffective in field applications for remedial or preventive control of bed bugs. Rather, each individual control scenario will likely dictate the choice of insecticide, the manner of application, and the level of control afforded by multiple integrated tactics employed by a pest management professional. Careful rotations of insecticides, as has been a relatively common practice for any applicator attempting to sustain the use of an insecticide, and thorough and comprehensive applications will likely control bed bugs in most urban scenarios. However, the lack of registered insecticides that can be applied when poultry are present in infested facilities make bed bug management extremely difficult. The broiler-

breeder egg production cycle is generally 265 d long, thus, bed bug populations reach high numbers before the end of the egg production cycle. Due to the physical environment in the production facilities it is extremely difficult to effectively apply those compounds that are registered for use when the chickens are present. In addition, many avenues of infestations exist ranging from wild birds, poultry production workers moving from house to house as well as movement of egg shipping materials from farm to farm. The use of pyrethroids such as those reported in our present studies and future registration of new and novel insecticides such as imidacloprid, fipronil, spinosyn and bifenthrin, are all viable candidates for effective bed bug management in both urban and poultry production environments.

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# Early Season Production System for Soybeans: Influence on Bean Leaf Beetle (Coleoptera: Chrysomelidae) and Bean Pod Mottle Disease in Mississippi[1]

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**ABSTRACT** The influence of an early season production system (ESPS) on the population of bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae), adults and associated incidence of bean pod mottle (BPM) disease in soybeans was investigated in Mississippi. Early season production systems consist of planting maturity groups III or IV soybeans in mid-March to mid-April in the mid-South of the United States. Treatments in this study included early-planted and late-planted maturity group IV and maturity group V soybeans. Populations of adult BLB, a vector of bean pod mottle virus, were greater in ESPS in 2001, but no difference between planting dates was recorded in 2000. Incidence of BPM was greater in early-planted soybeans in 2000. The greater number of BLB adults in the early-planted soybeans did not result in a greater incidence of BPM disease in the ESPS in 2001. Yields in all treatments were significantly different with the greatest yield in the early-planted, maturity group V soybeans in 2000. In 2001, the greatest yield was obtained from late-planted, maturity group IV soybeans. The results presented herein suggest that to further evaluate relationships between bean leaf beetle vector populations and incidence of bean pod mottle disease in comparisons of conventional soybean production systems with ESPS, experiments should consider a wider range of soybean maturity groups and larger experimental plots to more effectively evaluate differences among soybean varieties and minimize beetle dispersal and spread of bean pod mottle disease.

**KEY WORDS** Soybeans, planting date, bean leaf beetle, bean pod mottle disease

For many years, soybean planting in the mid-South of the United States has involved planting maturity groups V through VIII in mid- to late May (Board 1996). This system resulted in low and static yields (Heatherly 1999). The low yields were primarily due to the effects of drought and high temperature common in this region from August through September (Heatherly 1999). This period of high environmental stress often occurs during the critical, seed-set stage of plant development in these soybean maturity groups and can result in diminished yield. A relatively recent crop production recommendation includes early planting of early maturity groups of soybeans and is referred to as the early season production system (ESPS)

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(Heatherly 1999). The ESPS involves planting maturity groups III or IV soybeans in mid-March to mid-April. Planting early and utilizing an early maturity group soybean allows the producer to harvest earlier than for soybeans planted in mid- to late May. The critical, seed-set development stage can then escape harmful environmental stresses and relatively high insect pest infestations.

Changing an agroecosystem may result in concomitant changes in the behavior of organisms within the system. Research has detailed the effect of ESPS on various arthropod populations (Bowers 1995, Heatherly 1999). The purpose of this study was to determine the effect of the ESPS system on populations of bean leaf beetle (BLB), *Cerotoma trifurcata* (Förster), and the associated incidence of bean pod mottle (BPM) disease.

## Materials and Methods

**2000.** Soybeans were planted using a John Deere 7300 MaxEmerge II planter on the Mississippi Agricultural and Forestry Experiment Station Plant Science Research Farm located in Oktibbeha County, Mississippi. Roundup Ready™ (RR) varieties included Pioneer 9492 RR, a maturity group IV soybean planted April 28 and May 24, 2000, and Pioneer 95B95 RR, a maturity group V soybean planted on the same dates. Planting dates for this year were later than recommended for the ESPS due to rainfall. The Roundup Ready characteristic represents a current soybean production practice in the mid-South (National Agriculture Statistics Service 2001). Individual research plots consisted of 12 rows, each 96.5 cm wide and 16.5 m long. The four treatments were established with four replications in a randomized complete block design.

Prior to soybean emergence, the study area was oversprayed with Dual™ (metolachlor, Ceiba-Geigy Corp., Greensboro, NC) herbicide at a rate of 2.8 L per hectare to control weeds. Roundup™ (glyphosate, Monsanto, Chesterfield, MO) herbicide was applied June 15 at a rate of 2.1 L per hectare when the early-planted soybeans were at the V6 stage (Fehr & Caviness 1977) of plant growth and when the late-planted soybeans were at the V3 stage.

Sampling for BLB adults from emergence through the V5 stage of plant growth was conducted by visual examination of individual plants in one row on each sample date. Special attention was directed to cotyledonous leaves because this is a preferred feeding location for the beetles. Three subsamples, each consisting of all plants within 3.6 m of row (10.4 $m^2$ total area) were taken weekly for 4 weeks. The sampled site within each plot was marked by placing a stake in the soil in order to prevent repeated sampling of the same area. Border rows (two at each side of each plot) were excluded from sampling to minimize the effect of insect movement between plots. A random number generator was used to determine the row to be sampled excluding rows sampled in the three previous weeks. The number of BLB adults in each subsample was recorded. Soybean plots were first sampled on June 7.

A beat sheet sampling procedure (Kogan et al. 1980) was used each week beginning at V6 and continuing through the R8 stage of plant development because plants were larger and more difficult to examine with accuracy using the visual method. Three subsamples consisting of 3.6 m of row (10.4 $m^2$ total area) each were taken within the predetermined sample row. As with visual observations, the area in the row that was sampled was marked to minimize resampling the same

area. Due to limited plot size, resampling a marked area did occur but was avoided for 4 weeks. The area to be sampled was selected using the same procedure as for visual sampling. Samples were bagged, transported to the laboratory, and the number of BLB adults in each subsample was recorded. On July 5 sampling was conducted by both visual and beat sheet sampling methods and methods were compared to determine if significant difference existed between their efficacies. No significant difference in sampling efficacy was observed.

Plants within one row (16.5 m) in each plot with apparent symptoms of BPM, including chlorosis and mottling, were visually identified and recorded weekly throughout the growing season. Characteristic mottling of leaves in the upper canopy (top 2–3 nodes) caused by bean pod mottle virus (BPMV) (Windham & Ross 1985) was used to visually identify the disease. Observations were made weekly from June 7 to September 15 on plants within the same row sampled for beetles.

To confirm the accuracy of identifying BPM diseased plants in the field, soybean leaves were harvested from the upper two nodes of plants visually identified as positive or negative for symptoms of the disease. The leaf samples were tested for the presence of BPMV using enzyme linked immuno-sorbent assay (ELISA) (AgDia, Elkhart, IN). Ten symptomatic and ten asymptomatic plants were tested. Because symptoms of soybean mosaic (SM) disease may appear similar to those of BPM on soybeans, plants were also tested for soybean mosaic virus using ELISA procedures.

Yield measurements were taken by harvesting the middle four rows (66.0 m) of each plot. Plots were harvested using an MXP four-row plot harvester when the seed was at 12–13% moisture. Due to variance in moisture levels, not all treatments were harvested on the same date. The seed was bagged and taken to the laboratory. Because samples from each plot contained a large amount of trash, each sample was hand cleaned to remove foreign material. Seed weight was measured using a standard scale.

Data were analyzed by Proc GLM using SAS v. 8.2 (SAS Institute 2002). Analysis of variance and correlation procedures were conducted on numbers of bean leaf beetles and BPM symptomatic plants. Fisher's protected LSD was used to determine significant differences between numbers of bean leaf beetles, diseased plants, and yield among treatments.

**2001.** Soybean plots in 2001 were located in the same field as in 2000 and treatments included early and late plantings of the same varieties and maturity groups of soybeans. Soybeans were planted on April 10 and May 10 in plots consisting of 20 rows, each 16.5 m long, using the same equipment as in 2000. Planting dates in 2001 were as recommended for the ESPS. Four treatments, as in 2000, with four replications each were established in a randomized complete block design. Dual herbicide was applied prior to plant emergence as in the 2000 study, and Roundup was applied at the V7 growth stage in early-planted plots and at the V4 growth stage in late-planted plots.

The visual sampling procedure used to record BLB infestations in the 2000 study was repeated beginning May 4 and continued through the V5 growth stage. Samples were taken from 16.5 m (15.2 $m^2$ total area) of row (one row in each plot) and sample sites were determined as in 2000. In the 2001 study, samples were taken weekly for 3 weeks and the area sampled was marked to prevent resampling of the same area. After the V5 growth stage, plants were sampled

using the sweep net technique (Kogan et al. 1980) sampling a continuous length of row. Thirty-six sweeps (13.2 $m^2$ total area) were taken weekly from June 8 to September 13. Sweep net samples were bagged, transported to the laboratory, and the number of BLB adults in each sample was recorded. On June 8 sampling was conducted by both visual and sweep net sampling methods to again determine if significant difference existed between sampling efficacies. No significant difference in sampling efficiency was observed.

Plants within one row (16.5 m) in each plot with symptoms of BPM were visually identified and recorded weekly from May 4 to September 7. Samples were analyzed using ELISA to confirm the precision of sampling for infected plants in the field. As in 2000, soybean leaf samples were tested for the presence of both BPMV and SMV. Yield measurements were taken from each plot as in 2000 and data were analyzed using SAS v 8.2.

## Results and Discussion

There was no significant correlation between the numbers of adult BLB and soybean plants infected with BPM when data were analyzed within sample dates or by seasonal totals ($r = -0.09661$ (2000) and $r = -0.01084$ (2001)). Data for populations of BLB and numbers of BPM diseased plants will be discussed independently.

**2000.** Bean leaf beetle adults were not observed on the early-planted soybeans until June 7, 2000 (day 40 after planting), at which time they were in low numbers (Fig. 1). The soybean plots were located adjacent to a forest border,

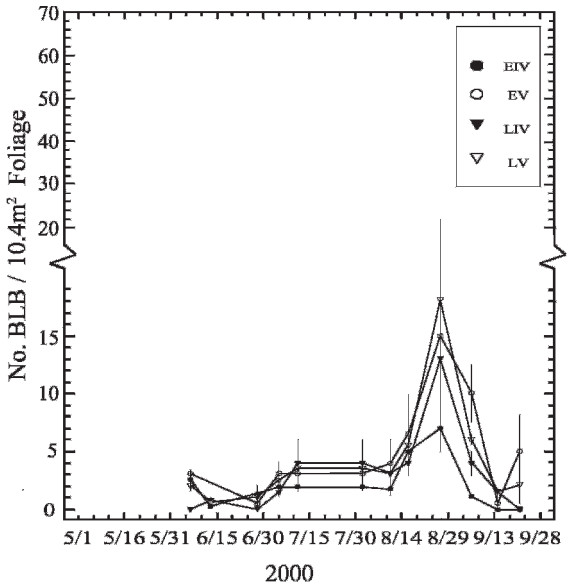


**Fig. 1.** Bean leaf beetle (BLB) population levels in early-planted maturity group IV (EIV), early-planted maturity group V (EV), late-planted maturity group IV (LIV), and late-planted maturity group V (LV) soybeans in Oktibbeha Co., Mississippi. 2000.

**Table 1. Seasonal total numbers of adult bean leaf beetle adults and bean pod mottle diseased plants in soybean for the main effects of early-planted, late-planted, maturity group IV, and maturity group V treatments, and yields for the four planting date X maturity group combinations in Oktibbeha Co., Mississippi. 2000.**

| Main effect | Bean leaf beetles per 10.4 $m^2$ foliage[1] | BPM diseased plants per 16.5 m row[1] | Yield (kg/ha)[1] Maturity Group IV | Yield (kg/ha)[1] Maturity Group V |
|---|---|---|---|---|
| Planting date | | | | |
| Early | 51±6 a[2] | 166±5 a | 169±10 c | 422±10 a |
| Late | 56±6 a | 110±5 b | 95±10 d | 296±10 b |
| Maturity group | | | | |
| IV | 49±6 a | 142±5 a | — | — |
| V | 58±6 a | 134±5 a | — | — |

[1]Means within a column for beetles or BPM and among yields for maturity group and planting date followed by the same letter are not significantly different ($P < 0.05$).
[2]Least square means (±SE).

which is an ideal overwintering site for BLB adults (Pedigo 1994). However, the late-planted soybeans in 2000 were not above ground when overwintering BLB moved from overwintering sites. These beetles moved to early planted soybeans in the area. Initial measurements of BLB adults were 2.3 ± 1 and 2.8 ± 1 adults per 10.4 $m^2$ of foliage in early-planted maturity group IV and V soybeans, respectively. Numbers of adult beetles remained at low levels (fewer than 10 beetles per 10.4 $m^2$) through mid-August. No significant differences in adult BLB populations were recorded for planting date treatments until September 6, at which time maturity group IV soybeans had begun senescence and higher adult populations were recorded in early-planted maturity group V plots. Bean leaf beetle adults move from more mature soybeans to less mature soybeans, indicating a preference for new plant growth (Pedigo & Zeiss 1996).

Seasonal cumulative numbers of BLB adults in treatment plots indicated that there were no significant effects of maturity group or planting date on beetle infestation ($F$: 0.49, df = 1,12, $P$ = 0.4953 and $F$: 1.18, df = 1,12, $P$ = 0.29807, respectively) (Table 1). Interaction between maturity group and planting date was also not significant ($F$ = 1.18, df = 1, 12, $P$ = 0.3617). The somewhat greater number of beetles recorded in maturity group V plots than in maturity group IV plots can be accounted for by the relatively large number of beetles moving into these plots from the earlier maturing group IV soybeans.

Symptoms of BPM disease (Windham & Ross 1985) were first observed on June 28, 46 days after planting (Fig. 2). There were no statistical differences in the initial measurements of the number of symptomatic plants among treatments ($F$ = 0.74, df = 3, 12, $P$ = 0.5494). One might expect to observe the number of plants expressing BPM symptoms in early-planted soybean plots to be greater due to increased expression of disease symptoms on plants in cooler weather to

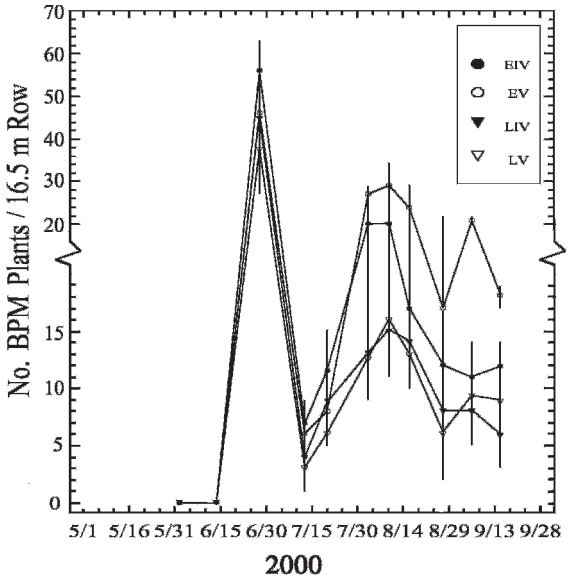


**Fig. 2.** Incidence of bean pod mottle (BPM) diseased plants in early-planted maturity group IV (EIV), early-planted maturity group V (EV), late-planted maturity group IV (LIV), and late-planted maturity group V (LV) soybeans in Oktibbeha Co., Mississippi. 2000.

which the early-planted soybean were subjected and due to the greater length of time for symptoms to develop on infected plants. By August 3, early-planted, maturity group V soybeans had significantly greater numbers of symptomatic plants than late-planted, maturity group V soybeans ($F = 4.35$, df = 3, 12, $P = 0.027$). This was also observed on September 15 ($F = 9.14$, df = 3, 12, $P = 0.002$) (Fig. 2). Reproductive development of soybeans is initiated later in group V soybeans than in earlier maturity groups in response to photoperiod (Fehr & Caviness 1977). Replication of the virus in soybeans is greatest during periods of vegetative growth, therefore the virus is afforded a longer period of time for propagation in later soybean maturity groups (Windham & Ross 1985).

Total numbers of BPM diseased plants observed throughout the sampling period indicated that a significantly greater number of diseased plants were observed in early-planted soybeans as compared with late-planted soybeans ($F = 69.9$, df = 1, 12, $P < 0.0001$) (Table 1). Interaction between main effects was not significant ($F = 2.90$, df = 1, 12, $P = 0.1145$).

Testing by ELISA for recognition of disease symptoms in the field showed a 100% level of accuracy in identifying BPM infected plants and a 90% level of accuracy in identifying non-infected plants; no plants tested positive for SMV.

Yield in treatment plots in 2000 was significantly different for all treatments ($F = 200.32$, df = 3, 12, $P = < 0.0001$) (Table 1). The greatest yield was obtained from early-planted maturity group V soybeans, followed by late-planted, maturity group V, early-planted maturity group IV, and late-planted maturity group IV soybeans. In research conducted in Texas (Bowers 1995) and in Mississippi (Heatherly 1996), greater yields were obtained in early-planted soybeans compared with late

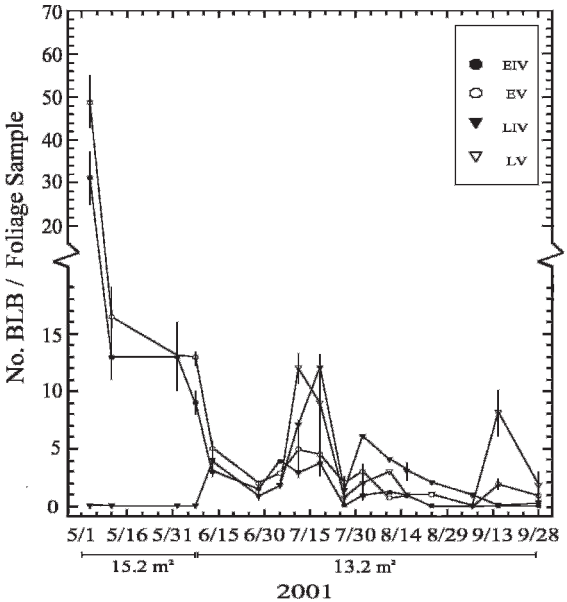


**Fig. 3.** Bean leaf beetle (BLB) population levels in early-planted maturity group IV (EIV), early-planted maturity group V (EV), late-planted maturity group IV (LIV), and late-planted maturity group V (LV) soybeans in Oktibbeha Co., Mississippi. 2001.

plantings. In the present study, the interaction between maturity group and planting date was significant for yield ($F = 6.61$, df = 1, 12, $P = 0.025$). Although greater numbers of diseased plants were recorded in early-planted, maturity group V soybeans, this level of disease did not result in a significant yield reduction. As the numbers of adult BLB did not significantly differ among treatments and did not reach economic injury levels (Pedigo 1994), the major factor impacting yields was apparently the influence of drought as described by Heatherly (1999).

**2001.** Unlike in 2000, BLB adults were observed on soybeans soon after plant emergence (day 24) in the early planted plots (Fig. 3). Numbers of BLB in all treatments, except for late-planted, maturity group IV soybeans, were much greater in soybean plots at this time than in 2000. Plots were planted earlier in 2001 than in 2000, probably accounting for the higher initial numbers of BLB adults. Because plots were placed adjacent to a forest border, beetles emerging from this overwintering site were drawn directly to the early-planted soybean plots rather than dispersing to other soybean fields in the area as appeared to have occurred in 2000 because of the delayed plantings. Early-planted, maturity group IV and V soybeans had significantly greater numbers of adult BLB than late-planted maturity group IV and V soybeans ($F = 35.36$, df = 3, 12, $P = < 0.0001$). The initial population of adult beetles decreased in both maturity group IV and V soybeans after the initial sampling week. This decline may be due to adult dispersal to nearby soybean fields or mortality of some beetles. Mortality of BLB in the overwintering generation is reported to occur shortly after beetle emergence (Schumm et al. 1983). Initial observations of BLB in late-planted

**Table 2. Seasonal total numbers of bean leaf beetle adults and bean pod mottle (BPM) diseased plants and yield for early-planted maturity group IV (EIV), late-planted maturity group IV (LIV), early-planted maturity group V (EV), and late-planted maturity group V (LV) soybeans in Oktibbeha Co., Mississippi. 2001.**

| | Bean leaf beetles per 15.2 $m^2$ foliage[1] | BPM diseased plants per 16.5 m row[1] | Yield (kg/ha) |
|---|---|---|---|
| EIV | 82 ± 8 b[2] | 43 ± 29 b | 483 ± 31 b |
| EV | 123 ± 8 a | 64 ± 29 ab | 522 ± 31 b |
| LIV | 47 ± 8 c | 143 ± 29 a | 658 ± 31 a |
| LV | 48 ± 8 c | 120 ± 29 ab | 494 ± 31 b |

[1]Means followed by the same letter are not significantly different ($P < 0.05$).
[2]Least square means (±SE).

soybeans were made on June 13. At this time, no significant differences in beetle numbers were observed among treatment plots.

Significant differences in adult BLB populations were recorded on July 19 and September 13. In general, the greatest number of BLB were recorded in late-planted, maturity group IV and early-planted maturity group V soybeans (Fig. 3). These differences were recorded until the maturity group IV soybeans began to dry around late August at which time the beetles moved to the more attractive maturity group V soybeans.

The seasonal cumulative number of adult BLB observed in plots was compared for treatments as in 2000 (Table 2). Interaction between maturity group and planting date was significant for planting date ($F = 6.68$, df = 1, 12, $P = 0.0239$). The greatest numbers of beetles were recorded in early-planted, maturity group V soybeans, followed by early-planted, maturity group IV, and late-planted maturity groups IV and V. These data support previous reports of greater levels of BLB infestations in early-planted compared with late-planted soybeans (Pedigo & Zeiss 1996).

BPM disease symptoms were first observed in soybean plots on June 1 (Fig. 4). The number of diseased plants per 15.8 m of row on this date in early-planted, maturity group IV soybeans (14 ± 2) was significantly greater than the number of diseased plants in early-planted, maturity group V soybeans (8 ± 1) ($F = 35.4$, df = 3, 12, $P = < 0.0001$). This difference may be partially explained by the more rapid development of maturity group IV soybeans making them more attractive to the adult beetles than the maturity group V soybeans. However, the difference in adult BLB numbers did not persist past the first week of sampling.

Bean pod mottle disease symptoms were not observed in late-planted soybeans until June 28 (Fig. 4). The number of diseased plants in these plots was similar to that recorded in early-planted, maturity group V soybeans. The early-planted, maturity group IV soybeans had the highest level of diseased plants for 2 weeks, after which numbers of plants expressing symptoms increased in late-planted,

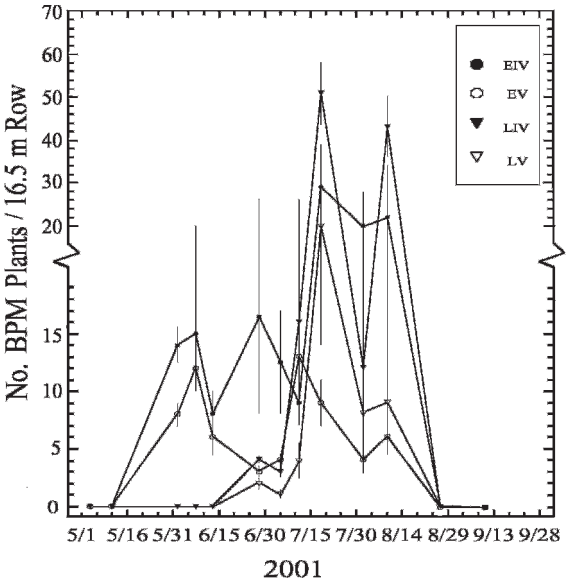


**Fig. 4.** Incidence of bean pod mottle (BPM) diseased plants in early-planted maturity group IV (EIV), early-planted maturity group V (EV), late-planted maturity group IV (LIV), and late-planted maturity group V (LV) soybeans in Oktibbeha Co., Mississippi. 2001.

maturity group V soybeans. This pattern persisted until the end of the sampling period in early August.

Total numbers of plants expressing BPM symptoms over all sample dates for each treatment were calculated as in 2000 (Table 2). Interaction between maturity group and planting date was significant ($F = 7.13$, df = 1, 12, $P = 0.0204$).

Testing by ELISA for recognition of disease symptoms in the field in 2001, as in 2000, showed a 100% level of accuracy in identifying BPM infected plants and a 90% level of accuracy in identifying non-infected plants; no plants tested positive for SMV.

Yield from late-planted, maturity group IV soybeans was greater than that from other plantings ($F = 6.63$, df = 3, 12, $P = 0.0069$). Interaction between maturity group and planting date was significant ($F = 10.45$, df = 1, 12, $P = 0.0072$).

Although BLB adults were in greater numbers in soybean plots planted early in ESPS, the plants in these plots did not have a greater incidence of BPM disease compared with soybeans planted one month later in one of two years in this study. Factors such as rainfall must be considered in close examination of these results. One of the principle factors resulting in low yields for conventional soybean plantings is drought during August and September. Below average rainfall levels were recorded for these months in 2000, but not in 2001 when rainfall for these months was above average (MAFES, unpublished). With similar research, it would be useful to use more widely varying soybean maturity groups to better separate the effects of early soybean production from the conventional use of later plantings of later maturity groups of soybeans, particularly in the

Southern United States. Furthermore, as BLB adults are capable of dispersing considerable distances, the size of the treatment plots can be an important factor in measuring the influence of this vector of BPM virus in soybeans. Plot size in the current study could have been larger to minimize the movement of bean leaf beetles from plot to plot.

## Acknowledgments

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# Early Season Production System for Soybeans: Influence on Bean Leaf Beetle (Coleoptera: Chrysomelidae) and Bean Pod Mottle Disease in Mississippi[1]

Ronald C. Stephenson and Henry N. Pitre[2]

J. Agric. Urban Entomol. 25(1): 53–62 (January 2008)

**ABSTRACT** The influence of an early season production system (ESPS) on the population of bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae), adults and associated incidence of bean pod mottle (BPM) disease in soybeans was investigated in Mississippi. Early season production systems consist of planting maturity groups III or IV soybeans in mid-March to mid-April in the mid-South of the United States. Treatments in this study included early-planted and late-planted maturity group IV and maturity group V soybeans. Populations of adult BLB, a vector of bean pod mottle virus, were greater in ESPS in 2001, but no difference between planting dates was recorded in 2000. Incidence of BPM was greater in early-planted soybeans in 2000. The greater number of BLB adults in the early-planted soybeans did not result in a greater incidence of BPM disease in the ESPS in 2001. Yields in all treatments were significantly different with the greatest yield in the early-planted, maturity group V soybeans in 2000. In 2001, the greatest yield was obtained from late-planted, maturity group IV soybeans. The results presented herein suggest that to further evaluate relationships between bean leaf beetle vector populations and incidence of bean pod mottle disease in comparisons of conventional soybean production systems with ESPS, experiments should consider a wider range of soybean maturity groups and larger experimental plots to more effectively evaluate differences among soybean varieties and minimize beetle dispersal and spread of bean pod mottle disease.

**KEY WORDS** Soybeans, planting date, bean leaf beetle, bean pod mottle disease

For many years, soybean planting in the mid-South of the United States has involved planting maturity groups V through VIII in mid- to late May (Board 1996). This system resulted in low and static yields (Heatherly 1999). The low yields were primarily due to the effects of drought and high temperature common in this region from August through September (Heatherly 1999). This period of high environmental stress often occurs during the critical, seed-set stage of plant development in these soybean maturity groups and can result in diminished yield. A relatively recent crop production recommendation includes early planting of early maturity groups of soybeans and is referred to as the early season production system (ESPS)

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# Susceptibility of the Bed Bug *Cimex lectularius* L. (Heteroptera: Cimicidae) Collected in Poultry Production Facilities to Selected Insecticides[1]

C. Dayton Steelman,[2] Allen L. Szalanski,[2] Rebecca Trout,[2] Jackie A. McKern,[2] Cesar Solorzano,[2] and James W. Austin[3]

J. Agric. Urban Entomol. 25(1): 41–51 (January 2008)

**ABSTRACT** *Cimex lectularius* L. is a widespread hematophagus insect pest around the world and is currently experiencing a reemergence as a public health pest of concern. One possible source of bed bugs to the human environment is the movement of bed bugs from poultry facilities to human structures by poultry workers. No recent studies have been conducted on the susceptibility of this insect to a wide range of insecticides. In addition, populations of bed bugs from poultry facilities have not been screened against insecticides for over 15 yr. Adult bed bugs collected from three poultry facilities in northwest Arkansas were exposed for 24 or 48 h (25°C) to glass vials treated with various dilutions of 12 insecticides dissolved in acetone to determine the concentration–response relationship. The order of toxicity, from most to least based on the $LC_{50}$s was: λ-cyhalothrin, bifenthrin, carbaryl, imidacloprid, fipronil, permethrin, diazinon, spinosyn, dichlorvos, chlorfenapyr, and DDT. Significant differences in $LC_{50}$ and $LC_{90}$ values for diazinon was observed among the three populations due to the previous history of repeated exposure to a mixture of tetrachlorvinphos and dichlorvos over a 10 yr period when compared to the $LC_{50}$s of two populations that had been exposed to the tetrachlorvinphos and dichlorvos mixture during 2–3 flock cycles. Bed bugs in each of the three populations exhibited high levels of DDT resistance, $LC_{50} >$ 100,000 ppm, which confirms that resistance to this insecticide continues in bed bug populations. This study documents baseline toxicological data for 12 insecticides in three populations of bed bugs and provides the first data on bed bug susceptibility to fipronil, spinosyn, and imidacloprid.

**KEY WORDS** bed bug, *Cimex lectularius*, insecticide resistance

*Cimex lectularius* L. (Heteroptera: Cimicidae) is a hematophagous insect that can be a major pest in breeder poultry facilities (Axtell 1985) and has regained worldwide attention due to its recent resurgence into dwellings shared by humans. Both sexes feed on blood and require a blood meal for subsequent molts (Usinger 1966). Although active dispersal of bed bugs can be important, passive dispersal is almost exclusively their dispersal *modus operandi*. This species is easily translocated by passive dispersal and adapts to multiple hosts (Usinger 1966, Marshall 1981, Lehane 2005). Consequently, when host animals including

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# Molecular Phylogeography of the Subterranean Termite *Reticulitermes tibialis* (Isoptera: Rhinotermitidae)[1]

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J. Agric. Urban Entomol. 25(2): 63–79 (April 2008)

**ABSTRACT** A molecular genetics study involving DNA sequencing of a portion of the mitochondrial DNA 16S gene was undertaken to determine the extent of genetic variation within the subterranean termite, *Reticulitermes tibalis*, in the United States. A total of 106 samples were analyzed from 18 states. Thirty-two nucleotide sites were variable in the 428 bp 16S rDNA sequence, and 41 distinct haplotypes were observed. Twenty-three haplotypes (55%) occurred only once, while the most common haplotype, T2, occurred in 24% of the samples. Genetic diversity among haplotypes ranged from 0.2 to 2.3%. Bayesian phylogenetic and TCS spanning tree analysis revealed several distinct clades that appeared to be geographically isolated; however, regression and Mantel tests did not support any populational structure. The role of glaciation and sky islands on haplotype variation of *R. tibialis* is supported by a molecular clock and may have contributed to the large amount of genetic variation observed within this species.

**KEY WORDS** mitochondrial DNA, DNA sequence, genetic variation, *Reticulitermes*, termite

The majority of pestiferous subterranean termites in North America belong to the endemic genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Reticulitermes* species are found in every state in the continental United States except Alaska, but are most common in the warm and humid southeastern region (Su et al. 2001). There are presently six described species of *Reticulitermes* in North America, four of which occur in western United States: the eastern subterranean termite, *Reticulitermes flavipes* Kollar, the light southern subterranean termite *R. hageni* Banks, the western subterranean termite, *R. hesperus* Banks, and the arid subterranean termite, *R. tibialis* Banks (Banks 1920, Snyder 1954, Weesner 1965, Messenger 2003). There is also genetic evidence for a new species of *Reticulitermes* in western United States and British Columbia, Canada (Szalanski et al. 2006).

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The arid subterranean termite, *R. tibialis*, is found from California to eastern Texas, and as far north as Iowa to Idaho (Banks & Snyder 1920, Snyder 1954, Weesner 1965, Nutting 1990). An isolated population has also become established in sand dunes located in Indiana (Park 1929, Ye et al. 2004).

This species is an economic pest in western United States. In its natural habitat, it can cause a small amount of damage to roots and stems of plants. It can also cause economic damage to man made structures. This termite is capable of building shelter tubes up to wooden structures; however, it is much less persistent in building tubes than other *Reticulitermes* species. When it does attack structures, it is more likely to attack moist and decaying wood than dry lumber (Pickens 1934), and this may be due to its preference for wood with high humidity (Williams 1934).

Genetic variation for a species is best determined if sampling from populations is distributed within the species range (Mayr & Ashlock 1991). Given the known distribution of *R. tibialis* in North America, the objective of this study was to document the extent of its genetic variation in North America. Avise (1994) suggests that smaller scale investigations may not provide a discernable population structure which might be more easily observed with larger scale studies. To our knowledge, this is the first comprehensive attempt to determine the extent of genetic variation for *R. tibialis* in North America, and the first study which encompasses most of the known geographic distribution of the species.

## Materials and Methods

Termites were collected from various locations in the United States from our own collecting efforts, from samples provided by collaborators, and from the 2002 National Termite Survey (Fig. 1). With the exception of the samples from Utah and Texas, only one to three samples were analyzed from the remaining states. All samples collected from the field were preserved in 90–100% ethanol. *Reticulitermes* were morphologically identified to species when alates were available using the keys of Krishna & Weesner (1969), and from labral measurements of soldiers (Hostettler et al. 1995, Heintchel et al. 2006) and other morphometrics outlined by Light (1927) and compiled by Castle (1930). For the remaining samples, species identification was conducted using mtDNA 16S sequences (Szalanski et al. 2003). Voucher specimens preserved in 100% ethanol are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

Alcohol-preserved specimens were dried on filter paper, and DNA was extracted according to Liu & Beckenbach (1992) on individual whole worker termites with the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μl of Tris:EDTA and stored at −20°C. Polymerase chain reaction was conducted using the primers LR-J-13007 (5′-TTACGCTGTTATCCCTAA-3′) (Kambhampati & Smith 1995) and LR-N-13398 (5′-CGCCTGTTTATCAAAAACAT-3′) (Simon et al. 1994). These PCR primers amplify an approximately 428 bp region of the mtDNA 16S rRNA gene. The PCR reactions were conducted with 1 μl of the extracted DNA (Szalanski et al. 2000), having a profile consisting of 35 cycles of 94°C for 45 s, 46°C for 45 s and 72°C for 60 s. Amplified DNA from individual termites was purified and concentrated with minicolumns (Wizard PCRpreps, Promega, Madison, WI)

**Fig. 1.** Genealogical relationship of *R. tibialis* mtDNA haplotypes. Each circle/node represents a single nucleotide substitution. Haplotypes T1 and T4 were collected from type locality specimens in Beeville, TX 2005, and haplotypes 43–46 from Missouri are not presented.

according to the manufacturer's instructions. Samples were sent to the University of Arkansas Medical Center DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions. Additional *R. tibialis* mtDNA 16S sequences were obtained from previous studies of *Reticulitermes* from Texas (Austin et al. 2004a), California (Tripodi et al. 2006) and Indiana (Ye et al. 2004). GenBank accession numbers were FJ610446 to FJ610472 for the new *R. tibialis* haplotypes found in this study. Consensus sequences for each sample were obtained using Bioedit 5.09 (Hall 1999). Mitochondrial DNA haplotypes were aligned using MacClade v4 (Sinauer Associates, Sunderland, MA). Genealogical relationship of *R. tibialis* haplotypes was constructed using TCS (Clement et al. 2000).

The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution (Kimura 1980). Mitochondrial 16S sequences from '*R. okanaganensis*', *R. hesperus* Banks, *R. malletei* Clément, *R. hageni* Banks, *R. flavipes* (Kollar) and *R. virginicus* (Banks) (Szalanski et al. 2003, Austin et al. 2004a,b,c, Szalanski et al. 2006, Austin et al. 2007) were added to the *R. tibialis*

dataset along with DNA sequences from the Formosan termite, *Coptotermes formosanus* Shiraki (GenBank AY558910), and *Heterotermes aureus* (Snyder) (GenBank AY280399), which were added to act as outgroup taxa. DNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). Maximum likelihood (ML) and maximum parsimony (MP) analysis on the alignments was conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as a fifth character state for the maximum parsimony analysis, and a Ti/Tv ratio of 2.77 was used for the ML analysis. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1000 resamplings using the Branch and Bound algorithm of PAUP* (Fig. 3).

For Bayesian analysis, the best-fitting nucleotide substitution model was chosen according to the general time reversible + gamma (GTR+G) model among 64 different models using the ModelTest v 3.7 (Posada & Crandall 1998) and PAUP* 4.0b10 (Swofford 2001) programs. Phylogenetic trees were obtained using Bayesian inference with the GTR+G model using Bayesian Evolutionary Analysis Sampling Trees (BEAST) v1.4.2 software (Drummond & Rambaut 2007). For Bayesian inference, four Markov chains run for $10^6$ generations with a burn-in of $2 \times 10^4$ were used to reconstruct the consensus tree.

To test for correlation between genetic similarity and geographical distances (Km) we applied a linear regression using JMP version 5.1 software (SAS Institute, Cary, NC). A Mantel test (Sokal 1979, Manly 1985, 1997, Epperson 2003) was conducted to ensure interdependence was not involved via PopTools Version 3.0.5 (Hood 2000) using a matrices correlation (Pearson r).

## Results and Discussion

The phylogeographic patterns of *Reticulitermes* have been reported for over 80 yr now, but given the high degree of confusion for species identification and the inability to recover all diagnostic castes, updated records which utilize more objective identification tools such as genetics are needed. This approach has been effective for interpreting the distribution and evolutionary history of this morphologically ambiguous genus. *Reticulitermes tibialis* from 18 U.S. states were subjected to DNA sequencing analysis (Fig. 1, Table 1). DNA sequencing of the 16S rRNA amplicon revealed an average amplicon size of 428 bp. The average base frequencies were A = 0.41, C = 0.23, G = 0.13, and T = 0.23. Among the 106 *R. tibialis* mtDNA 16S DNA sequences, a total of 32 nucleotide sites were variable (Table 2). Forty one distinct haplotypes (lineages) were observed (Tables 1 and 2), and intraspecific genetic divergence among these haplotypes ranged from 0.2% to 2.3%. Twenty-three haplotypes occurred only once, while the most common haplotype, T2, occurred in 23% of the samples (Tables 1 and 2).

Some haplotypes appeared to have some geographical isolation significance as evident from the TCS spanning tree analysis and the Bayesian phylogenetic analysis. We conducted a TCS spanning tree analysis on all 42 *R. tibialis* haplotypes (Fig. 2). Regression of genetic distances and spatial distances (Km) can be observed in Figure 4. Although TCS results appear to indicate the possibility of structure among *R.tibialis* populations, this was not statistically supported. There was a poor relationship between genetic and geographic distances ($r^2 = 0.291$, $P = 0.008$, from 83 (of 106 total) distinct populations >2 km distances apart each) as only 29% of the variance of genetic *p*-distances can be

attributed to changes in geographic distance and the linear relationship between them. In addition, in order to account for any possible interdependence associated with input variables assigned to matrices, a permutation test (Mantel Test) was performed. The Mantel test was conducted by applying the Mantel Test option of PopTools version 3.0.5, released 30 May 2008 that employed 10,000 permutations. The simple matrix correlation (Pearson r) between genetic and geographic distance was 0.061 ($P < 0.001$ with 10,000 permutations); consequently, the overall poor relationship of geographic structure as evidenced by more distant populations was even less supported than the linear regression value ($r^2 = 0.291$) (Fig. 4).

The basal haplotype, T23, was recovered from Salt Lake Co., Utah. Reports of problems with *R. tibialis* attacking both ornamental plantings and structures have been known for decades (Rees & Gaufin 1939) in Utah. Many distinct haplotype lineages were observed; however, most of these lineages were only a single nucleotide in divergence from other observed haplotypes. The exceptions were haplotypes T25 from Washington Co., Utah and T41 from Ada Co., Idaho (Fig. 2). Two haplotypes were recovered from the type locality, Beeville, TX. Haplotype T4 was also found from New Braunfels, TX, while haplotype T1 was recovered from three other locations in Texas.

Both northern and eastern populations of *R. tibialis* pose interesting questions into the possibilities of biotic radiation of the species; in particular, the possibility that some populations have followed glacial refugia patterns (Comes & Kardereit 1998) as has been proposed in other studies of the genus from both nearctic (Austin et al. 2006, McKern et al. 2007) and palearctic (Uva et al. 2004, Luchetti & Mantovani 2005) regions. Although some have proposed migration or accidental introductions of *Reticulitermes* into Canada and north central states (Myles 2004), it is clear that many have continuously occupied these areas (Park 1929, Emerson 1936) and that termite occurrences, due to synanthropic associations with man, may be more directly related to the incidence of urban sprawl and development of these areas.

Of the 438 characters used for the phylogenetic analysis, a total of 71 characters (16.2%) were parsimony informative. The maximum parsimony analysis resulted in a tree of a length of 271 and a confidence index value of 0.531. The maximum likelihood analysis resulted in a single tree with a –ln L value of 1492.37750. As expected, the MP, ML, and Bayesian phylogenetic analysis revealed that *R. tibialis* formed a sister clade with *R. hesperus*, which was a sister group to another western *Reticulitermes* termite, '*R. okananganensis*' (Szalanski et al. 2006) (Fig. 3).

Among western populations, Castle (1930) applied morphological comparisons of several samples of *R. tibialis* to distinguish it from other congeners such as *R. hesperus*. Furthermore, Castle (1930) attempted to distinguish *R. tibialis* from *R. humilis* (not to be confused with *R. humilis* var. *hoferi* which is now known to be *Heterotermes aureus*) and *R. tumiceps*, both of which have been synonymized with *R. tibialis* by Snyder (1954). Recent genetic investigations confirm the presence of an undescribed nearctic *Reticulitermes* that occupies a range consistent with *R. tibialis* occurring on the eastern range of the Sierra Nevada and Rocky mountains (Szalanski et al. 2006) but is not as broadly distributed. This is not surprising given that other cryptic *Reticulitermes* species are being identified (Austin et al. 2007). Castle (1930) evaluated *R. tibialis* workers and

**Table 1. *Reticulitermes tibialis* collection locations, haplotypes (frequency), and number of samples per U.S. state (n).**

| State (n) | County/Parish, haplotype(s) (n) | Lat/Long | |
|---|---|---|---|
| AZ (9) | Cochise T19 | 315555N | 1094432W |
| | Coconino T11 | 360500N | 1120803W |
| | Mohave T10, T12, T24(2) | 360500N | 1134603W |
| | Santa Cruz T19(2) | 313500N | 1103502W |
| | Yavapai T10 | 343030N | 1122233W |
| CA (6) | Los Angeles T9 | 340143N | 1174837W |
| | Riverside T9, T10 | 335503N | 1164714W |
| | San Bernadino T3(2), T10 | 342535N | 1171803W |
| CO (6) | Larimer T27 | 403507N | 1050504W |
| | Mesa T17, T18(3), T26 | 390350N | 1083302W |
| ID (2) | Ada T30, T41 | 434144N | 1162114W |
| IN (2) | Porter T33 | 413947N | 0870213W |
| | Tippicanoe T2 | 402500N | 0865231W |
| KS (5) | Butler T7 | 374700N | 0965001W |
| | Harvey T33(2) | 380005N | 0973031W |
| | Reno T33 | 380356N | 0975452W |
| | Russell T32 | 385317N | 0985114W |
| LA (1) | St. Mary T8 | 295003N | 0913448W |
| MO (4)* | Eureka | 383009N | 0903740W |
| | Green City | 401607N | 0925712W |
| | Springfield | 371255N | 0931754W |
| | St. Louis | 383741N | 0901131W |
| MT (1) | Yellowstone T37 | 454700N | 1083002W |
| ND** (1) | Golden Valley | 471727N | 1020353W |
| NE (1) | Cheyenne T2 | 410804N | 1025807W |
| NM (7) | Bernalillo T11 | 350913N | 1064042W |
| | Dona Ana T3, T13 | 321844N | 1064642W |
| | San Juan T12, T14 | 364341N | 1081307W |
| | Sandoval T12, T15 | 351419N | 1064002W |
| NV (1) | Clark T31 | 360223N | 1145855W |
| OK (7) | Delaware T7 | 363537N | 0944609W |
| | Greer T2 | 345219N | 0993015W |
| | Payne T5 | 360656N | 0970330W |
| | Texas T8 | 363543N | 1013812W |
| | Tulsa T8(2) | 361021N | 0955627W |
| SD (1) | Lyman T27 | 435435N | 1000331W |
| TX (40) | Aransas T2(5) | 280246N | 0970215W |
| | Bee T1(2), T4 | 282403N | 0974454W |
| | Brewster T42 | 302131N | 1033940W |
| | Carson T36 | 352044N | 1012250W |
| | Collin T1 | 331200N | 0963401W |
| | Comal T4 | 294211N | 0980728W |
| | Culberson T14 | 310223N | 1044951W |
| | Dallas T6 | 323523N | 0965125W |
| | Denton T1 | 330052N | 0970549W |
| | El Paso T3, T9(2), T34 | 314531N | 1062913W |
| | Goliad T8, T35, T39 | 284006N | 0972318W |

**Table 1. Continued.**

| State (n) | County/Parish, haplotype(s) (n) | Lat/Long | |
|---|---|---|---|
| | Henderson T7(2) | 321218N | 0955120W |
| | Hill T35 | 320101N | 0970801W |
| | Hudspeth T6 | 315607N | 1051201W |
| | Kinney T2 | 291838N | 1002504W |
| | Leon T6 | 312750N | 0960329W |
| | Llano T5, T35, T38 | 305301N | 0982821W |
| | Midland T2 | 315950N | 1020440W |
| | Palo Pinto T2 | 324502N | 0981801W |
| | Swisher T5 | 344437N | 1015117W |
| | Tarrant T1 | 324331N | 0971915W |
| | Travis T2 | 302944N | 0975524W |
| | Ward T2 | 313100N | 1030402W |
| | Wilson T2, T40(2) | 290801N | 0980922W |
| UT (16) | Duchesns T10 | 401758N | 1095920W |
| | Grand T2, T3, T10, T12, T16 | 383424N | 1093259W |
| | Salt Lake T11, T12(2), T19, T20, T21(2), T22, T23 | 404539N | 1115328W |
| | Washington T25 | 371502N | 1131505W |
| WY (1) | Park T10 | 443135N | 1090324W |

*Pinzon-Florian (2007).
**Sample morphologically identified but not sequenced.

paratype samples (alates and soldiers) provided by T. E. Snyder (USNM collection) and from other collected samples from California, Oregon, Washington, Arizona, and Mexico. Although Castle (1930) suggests there was little variation in morphological measurements, we find significant genetic variation across its range. Given the breadth of the collection at his disposal, it is unclear why Castle (1930) evaluated *R. tibialis* specimens from Tracy, CA instead of type material from Beeville, TX. One of the key elements described in Castle's thesis suggests gular measurements were significantly different, and might be supported by more recent morphological studies of the same type (Brown et al. 2005). However, these results can vary considerably as has been viewed with type locality material of *R. tibialis* recently evaluated (Heintschel et al. 2006), and depending on what characters are applied.

Recent investigations into the dispersal capabilities of Rhinotermitids such as *Coptotermes formosanus* (Messenger et al. 2005) imply that many studies underestimate just how far termites can migrate through natural dispersal events. Tethered experiments with *R. flavipes* suggest a flight distance of almost 500 m is possible (Shelton et al. 2006), and earlier observations of *R. virginicus* have demonstrated that alates can be collected at significant altitudes ranging from 6 to 1000 m (Glick 1939). Wind speed at or below 1.0 m/s has been shown to be one of the most important microenvironmental factors involved in dispersal flight activity (Leong et al. 1983). Species such as *R. virginicus* have been observed making dispersal flights from the tops of trees as opposed to *R. flavipes* near the ground (DeHeer & Vargo 2006). This type of ecological separation and establishment may dramatically influence dispersal capabilities of *R. tibialis*.

**Table 2. Variable nucleotide sites among 42 *R. tibialis* haplotypes (H).**

| H | 55 | 60 | 75 | 77 | 119 | 127 | 128 | 131 | 132 | 134 | 140 | 161 | 162 | 163 | 164 | 165 | 169 | 179 | 185 | 188 | 211 | 229 | 232 | 237 | 269 | 293 | 317 | 364 | 365 | 369 | 374 | 401 | n |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T1 | G | T | C | A | A | C | . | A | A | G | G | A | . | G | G | A | T | T | C | T | A | T | A | C | C | A | T | A | T | . | T | G | 4 |
| T2 | - | - | - | - | - | - | . | - | - | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 10 |
| T3 | - | C | - | - | - | - | . | - | - | A | - | - | . | - | - | - | - | - | - | - | - | C | - | - | - | - | - | C | A | . | - | - | 5 |
| T4 | - | - | - | - | - | - | . | - | - | A | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 2 |
| T5 | - | - | - | - | - | - | . | - | - | - | - | - | . | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | A | . | - | - | 2 |
| T6 | - | - | - | - | - | - | . | - | - | A | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | . | - | - | 1 |
| T7 | - | - | - | - | - | - | . | T | - | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | . | - | - | 4 |
| T8 | - | - | - | - | - | - | . | T | - | - | - | - | . | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | A | . | - | - | 4 |
| T9 | - | C | - | - | - | - | . | - | - | A | - | - | A | - | - | - | - | - | - | - | - | C | G | - | - | - | - | C | A | . | - | - | 3 |
| T10 | - | C | - | - | - | - | . | - | - | A | - | - | A | - | - | - | - | - | - | - | - | C | - | - | - | - | - | C | A | G | - | - | 7 |
| T11 | - | - | - | - | G | - | . | - | . | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | G | - | - | 3 |
| T12 | - | - | - | - | G | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 5 |
| T13 | - | C | - | - | - | - | . | - | - | A | - | - | A | - | - | - | - | - | - | - | - | C | - | - | - | - | - | C | A | . | - | - | 2 |
| T14 | - | - | - | - | - | - | . | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T15 | - | - | - | - | - | - | . | - | - | - | - | - | A | - | - | - | C | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T16 | - | - | - | - | G | - | . | - | . | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | . | - | - | 1 |
| T17 | - | - | - | - | G | - | . | - | . | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T18 | - | - | - | - | G | - | . | - | - | - | - | - | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 3 |
| T19 | - | - | - | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T20 | - | - | - | - | G | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | . | . | - | - | 1 |
| T21 | - | - | - | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | . | . | - | - | 2 |
| T22 | A | - | - | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | C | - | - | - | - | - | - | - | C | A | . | - | - | 2 |
| T23 | A | - | - | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T24 | A | - | - | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 2 |
| T25 | - | - | - | - | G | - | . | - | - | - | - | - | . | A | - | G | - | - | - | - | C | - | - | - | - | - | - | C | A | . | C | - | 1 |

**Table 2. Continued.**

| H | 55 | 60 | 75 | 77 | 119 | 127 | 128 | 131 | 132 | 134 | 140 | 161 | 162 | 163 | 164 | 165 | 169 | 179 | 185 | 188 | 211 | 229 | 232 | 237 | 269 | 293 | 317 | 364 | 365 | 369 | 374 | 401 | n |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T26 | - | - | - | - | - | A | . | - | . | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | T | - | - | C | A | . | - | - | 1 |
| T27 | - | - | - | - | - | - | . | - | - | - | - | - | A | - | A | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 2 |
| T28 | - | - | - | - | G | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | C | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T29 | - | - | - | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | C | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T30 | A | - | - | - | G | - | . | - | - | - | A | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T31 | A | - | T | - | - | - | . | - | - | - | - | G | . | - | - | G | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T32 | - | - | - | - | - | - | C | - | - | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | . | - | - | 1 |
| T33 | - | - | - | - | - | - | . | - | - | - | - | - | . | - | - | - | - | - | - | - | - | - | - | T | - | - | - | - | A | . | - | - | 4 |
| T34 | - | C | - | - | - | - | . | - | - | - | - | - | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | - | A | 1 |
| T35 | - | - | - | - | - | - | . | - | - | A | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | A | . | C | - | 1 |
| T37 | - | - | - | - | G | - | . | - | . | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | - | G | C | A | . | - | - | 1 |
| T38 | - | - | - | - | - | - | . | - | - | A | - | - | . | - | - | - | - | - | T | - | - | N | - | - | - | - | - | - | A | . | - | - | 1 |
| T39 | - | - | - | - | - | - | . | T | - | - | - | - | . | - | - | - | - | C | - | - | - | - | - | - | - | - | - | - | A | . | - | - | 1 |
| T40 | - | - | - | - | - | - | . | - | - | - | - | G | . | - | - | - | - | - | - | - | - | - | - | T | - | - | - | C | A | . | - | - | 2 |
| T41 | - | - | - | G | G | - | . | - | - | - | - | G | . | A | - | G | - | - | - | - | C | - | - | - | - | - | - | C | A | . | - | - | 1 |
| T42 | - | - | - | - | - | - | C | - | - | - | - | - | . | - | - | - | - | - | - | - | - | - | - | - | - | G | - | - | A | . | - | - | 1 |

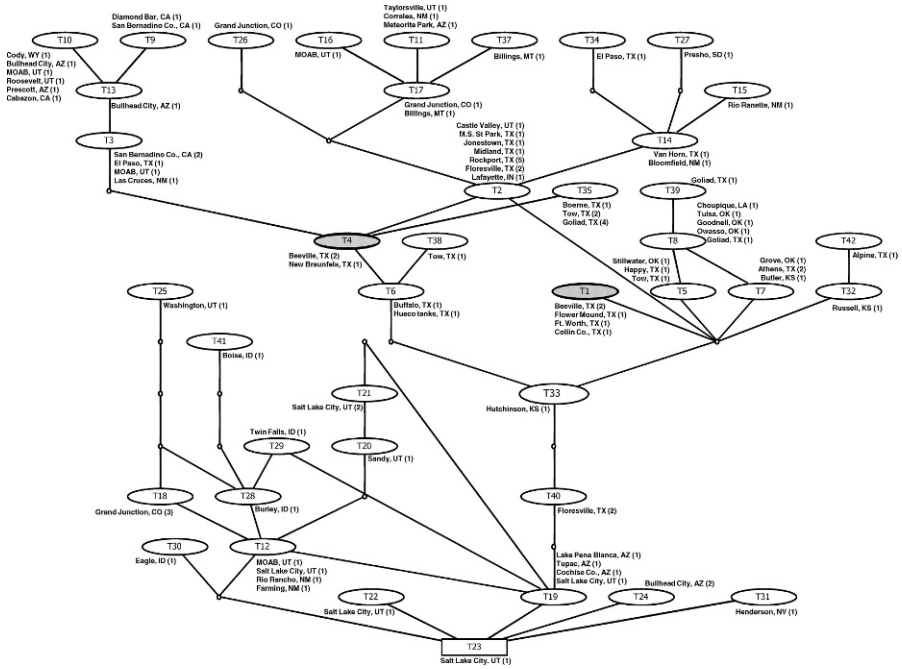


**Fig. 2.** Distribution of *Reticulitermes tibialis* haplotypes from the United States. Size of shaded circles is proportional to sample size, white circles are populations which were not used for genetic analysis.

Furthermore, the intense intraspecific competition of newly founded reproductives in close proximity to mature nests is also a consideration (Pickens 1934, Kitade et al. 2004, Green et al. 2006) which likely influences successful range expansion for this species, particularly since they are well established in more northerly locations, such as western Montana (Castle 1944), Idaho, Colorado, North Dakota (Austin et al. 2006), and likely extend well into Canada.

This is the first comprehensive genetic analysis of *R. tibialis* from the United States as a whole, and the phylogenetic relationships of this species provide potentially important information on the history of evolutionary change and range expansion of the species.

Because mitochondrial DNA is a single circular molecule which is apparently inherited only though females and does not undergo recombination, lacks complicating features such as introns and transposable elements, and evolves as much as ten to twenty times faster than most nuclear genes (Brown et al. 1979, Futuyma 1998), it is a highly efficient means to understanding the rate of mutation and provides valuable insight into historical radiation of *R. tibialis* in the Nearctic. Furthermore, the substitution rate commonly estimated for insects mtDNA, 2.3% genetic divergence / Myr (Brower 1994), was considered. Applying a molecular clock to all western congeners, we find 1.1% intraspecific genetic variation within *R. tibialis* with an estimated time of divergence approximating 478 kya. The interspecific genetic variation and estimated divergence of *R.*

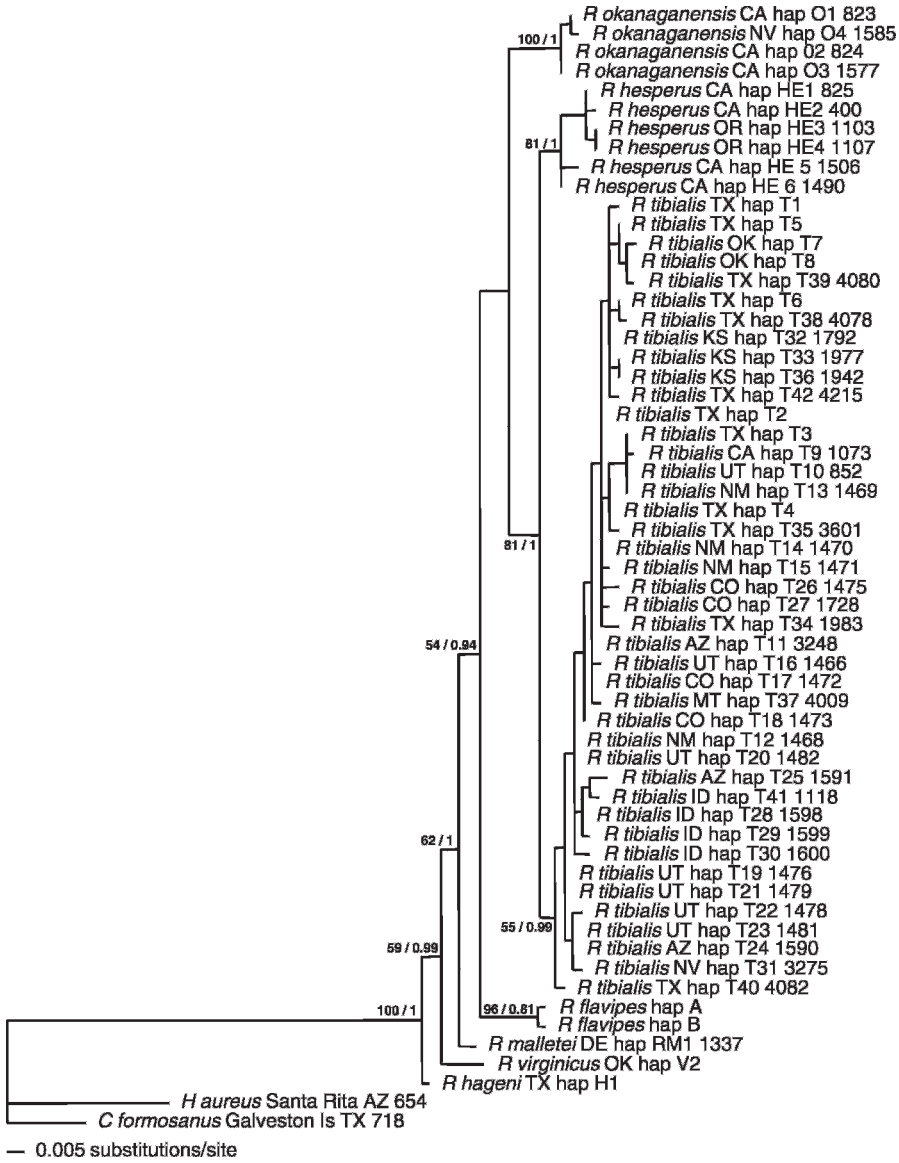


**Fig. 3.** Maximum likelihood phylogenetic tree of *Reticulitermes tibialis* haplotypes relative to other Rhinotermitidae termites. Maximum parsimony bootstrap values are provided and Bayesian posterior bootstrap values are listed nearest their respective branches right to left, respectively. Haplotypes 43–46 from Missouri, Courtesy of O. Pinzon-Florian are not presented (unpublished and unavailable on GenBank).

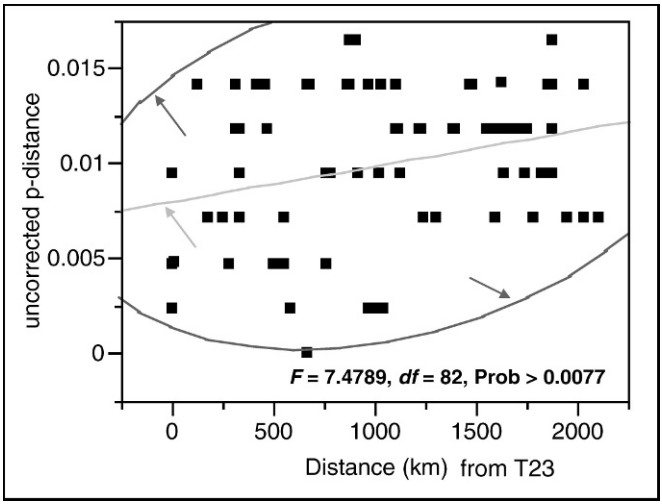


**Fig. 4.** Spatial relationships among *R. tibialis* in the United States applying a linear regression. Geographic distances from the most basal 16S rRNA haplotypes originating from Salt Lake City, Utah (T23) are on the X-axis; Uncorrected P-distances for all 46 *R. tibialis* haplotypes are on the y-axis. The Bivariate correlation and associated 2-tailed probability are listed just above the x-axis, with the light grey arrow pointing to the linear fitted line and red elipse (demarked by dark grey arrows) representing the 0.95 probability.

*tibialis* from other congeners was 2.1% (913 kya) for *R. hesperus*, and 2.8% (1.217 mya) for *R. okanaganensis*, respectively. Spatially and temporally distinct zones where ice sheets have receded and subsequently encroached over the last ice age likely play significant roles (Comes & Kardereit 1998) in the fragmentation, isolation and genetic divergence of this genus in the Nearctic, as have been proposed for populations of *R. hesperus* in British Columbia (Spencer 1937, 1945), the isolation of *R. okanagensis* from western populations of *R. hesperus* (McKern et al. 2007, Szalanski et al. 2006), and the likely eastern radiation of *R. tibialis* across the American Great Plains following glacial events (Austin et al. 2006).

Genetic analysis revealed a large amount of genetic variation within *R. tibialis*, second only to *R. flavipes* (Austin et al. 2006). Variation in genes, as well as in environmental conditions, is translated by processes of development into variation in phenotypic characters, such as morphological features (Futuyma 1998), which provides some ideas for the morphological variation described in literature for this species.

While one goal of applying molecular markers such as 16S is to identify morphologically ambiguous samples (Szalanski et al. 2003), another might be to identify the source of introductions to non-native areas (Szalanski et al. 2004). By comprehensive sampling across broad geographic zones, as presented in this study, it affords others the opportunity to develop molecular diagnostics for future invasive termite species to the United States. This study is just a small step in a long journey towards this goal, but an important one as it represents the most current view of

this species known to date. This study also demonstrates that *R. tibialis* has a large amount of genetic diversity in western United States; a fact not surprising given the climatic and geographic conditions where this species exists.

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# Molecular Phylogeography of the Subterranean Termite *Reticulitermes tibialis* (Isoptera: Rhinotermitidae)[1]

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**ABSTRACT** A molecular genetics study involving DNA sequencing of a portion of the mitochondrial DNA 16S gene was undertaken to determine the extent of genetic variation within the subterranean termite, *Reticulitermes tibalis*, in the United States. A total of 106 samples were analyzed from 18 states. Thirty-two nucleotide sites were variable in the 428 bp 16S rDNA sequence, and 41 distinct haplotypes were observed. Twenty-three haplotypes (55%) occurred only once, while the most common haplotype, T2, occurred in 24% of the samples. Genetic diversity among haplotypes ranged from 0.2 to 2.3%. Bayesian phylogenetic and TCS spanning tree analysis revealed several distinct clades that appeared to be geographically isolated; however, regression and Mantel tests did not support any populational structure. The role of glaciation and sky islands on haplotype variation of *R. tibialis* is supported by a molecular clock and may have contributed to the large amount of genetic variation observed within this species.

**KEY WORDS** mitochondrial DNA, DNA sequence, genetic variation, *Reticulitermes*, termite

The majority of pestiferous subterranean termites in North America belong to the endemic genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Reticulitermes* species are found in every state in the continental United States except Alaska, but are most common in the warm and humid southeastern region (Su et al. 2001). There are presently six described species of *Reticulitermes* in North America, four of which occur in western United States: the eastern subterranean termite, *Reticulitermes flavipes* Kollar, the light southern subterranean termite *R. hageni* Banks, the western subterranean termite, *R. hesperus* Banks, and the arid subterranean termite, *R. tibialis* Banks (Banks 1920, Snyder 1954, Weesner 1965, Messenger 2003). There is also genetic evidence for a new species of *Reticulitermes* in western United States and British Columbia, Canada (Szalanski et al. 2006).

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# Efficacy of Entomopathogenic Viruses on Pickleworm Larvae and Cell Lines[1]

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**ABSTRACT** A critical need exists for new biological pest management techniques for the pickleworm, *Diaphania nitidalis* (Stoll) (Lepidoptera: Pyralidae), a major pest of Cucurbitaceae. One potential management strategy for this pest is the use of entomopathogenic viruses. In this study, several viruses were evaluated for efficacy against pickleworm cell lines and second-stage larvae. First, selected viruses were bioassayed *in vitro* against one suspended cell line (IPLB-DnEs1) and one attached cell line (IPLB-DnEa3) that we derived from pickleworm embryos. In these experiments, a multiply-embedded nucleopolyhedrovirus (AgMNPV) from velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, showed the greatest activity toward the pickleworm cell lines. Following these results, 14 entomopathogenic viruses in the families Baculoviridae, Reoviridae, and Iridoviridae were evaluated *in vivo* in bioassays of second instar *D. nitdalis*. Among these 14 viruses, only six baculoviruses showed significant activity against pickleworm larvae. AgMNPV and a multiply-embedded nucleopolyhedrovirus (AcMNPV) from alfalfa looper, *Autographa californica* (Speyer), were the most efficacious against second instar *D. nitidalis*. Moreover, five of six stilbene fluorescent brighteners significantly increased efficacy of AgMNPV against pickleworm larvae.

**KEY WORDS** baculovirus, AgMNPV, *Diaphania nitidalis*, Pyralidae, insect cell line

The pickleworm, *Diaphania nitidalis* (Stoll) (Lepidoptera: Pyralidae), is a serious pest of Cucurbitaceae (York 1992, Capinera 2001). Female pickleworm moths deposit eggs in small clusters on leaves and flower buds of cucumber, *Cucumis sativus* L.; cantaloupe, *Cucumis melo* L.; pumpkin, *Cucurbita pepo* L.; squash, *Cucurbita* spp.; and other cucurbits (Reid & Cuthbert 1956). Eggs hatch in 2–4 d, and young larvae initially feed close to where they hatch (Fulton 1947, Canerday & Dilbeck 1968). However, as larvae grow older they enter fruits, where they can cause severe economic damage (Fulton 1947). Control of the early instars is important because once the larvae penetrate the fruits, they are nearly impossible to kill with insecticides, and by then the fruits have been ruined and

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cannot be sold (Fulton 1947, Hughes et al. 1983). Larvae also may spin a silken web over the entrance hole, which may make them less susceptible to natural enemies or insecticide sprays (Reid & Cuthbert 1956). Other *Diaphania* species, such as the melonworm, *D. hyalinata* (L.) (Medina-Gaud et al. 1989, Guillaume & Boissot 2001, Gonring et al. 2003), and pumpkin caterpillar, *D. indica* (Saunder) (Peter & David 1991, Schreiner 1991), are also serious pests of cucurbit production worldwide (Whittle & Ferguson 1987, Clavijo et al. 1995, Bacci et al. 2006). In Florida and the Caribbean, melonworm can be even more damaging than pickleworms (Posada 1992).

Pickleworm larvae are especially destructive to pickling cucumbers because of the near zero tolerance for infestations (York 1992). This pest is often the number one production problem for cucurbit growers in the mid-Atlantic states. For example, losses due to pickleworms have been estimated to be 2–3 million dollars annually in Georgia alone (Sparks & Riley 2001). Because of the low threshold for pickleworm damage to many cucurbits (Hughes et al. 1983), crops are often sprayed on a schedule, whether or not the insects are actually present (Kaplan 1989). Even with heavy insecticide applications, acceptable control is not always achieved. Because of health and environmental concerns, the Food Quality Protection Act (FQPA) of 1996 restricts the total use of chemical pesticides on agricultural crops (EPA 2007). All pesticides with a similar mode of action must now be considered together in this "risk cup." Because most pesticides undoubtedly will be applied to the major crops, fewer materials will be left for fruits and vegetables, including cucurbits. Thus, there is a critical need for biologically related pest management tools for this important vegetable pest.

Natural enemies have been of limited benefit in suppressing pickleworm populations, and few serious attempts at introducing biological control agents have been made in the United States (Pena et al. 1987, Smith et al. 1994). Except for nematodes and *Bacillus thuringiensis*, there have been few control efforts of pickleworms with entomopathogens (Delplanque & Gruner 1975, Bennett & Capinera 1994, Shannag et al. 1994).

Entomopathogenic viruses are generally considered to be environmentally safe and do not leave toxic residues (Monobrullah 2003). However, widespread use of viruses for pest management has been hampered by their relatively high cost, slow activity, and susceptibility to UV degradation (Moscardi 1999). Of the approximately 15 families of viruses that infect invertebrates, the baculoviruses (Baculoviridae) are by far the most important for the development of pest management tools (Evans 2000). Other virus families that also have received attention for pest management applications include iridescent viruses (Iridoviridae) and cypoviruses (Reoviridae) (Anonymous 2007). Baculoviruses, Nucleopolyhedrovirus (NPV) and Granulovirus (GV) are generally regarded as safe and effective bioinsecticides against a wide range of lepidopteran pests (Moscardi 1999, Lacey et al. 2001). However, the only report that we found regarding baculoviruses and pickleworms was with the NPV of *Spodoptera albula* Walker (Lepidoptera: Noctuidae). This baculovirus is being marketed as VPN 82 and VPN Ultra (Agrcola El Sol, Guatemala City, Guatemala; http://www.agricolaelsol.com) for use against *D. nitidalis* and *D. hyalinata* in Nicaragua and Guatemala (Lecuona 2002). However, there is no published literature on the efficacy of these materials against *Diaphania* species.

Although the baculoviruses are generally characterized by their narrow host range, some NPVs have broader host ranges, especially the NPVs of *Anagrapha falcifera* (Kirby) (Lepidoptera: Noctuidae) and *Autographa californica* (Speyer) (Lepidoptera: Noctuidae), which have been reported to infect species in 10 and 12 insect families, respectively (Evans 2000). Since the early 1980s, the *Anticarsia gemmatalis* multicapsid nucleopolyhedrosis virus (AgMNPV) has been widely used in Brazil for biological control of the velvetbean caterpillar, *A. gemmatalis* Hübner (Lepidoptera: Noctuidae), on soybean (Moscardi & Sosa-Gómez 2000). Carner et al. (1979) found that of eight noctuid species tested, only *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) was particularly susceptible to AgMNPV. However, Evans (2000) reported the host range of AgMNPV to include nine species in two families, including Pyralidae (Pavan 1981, Ribeiro et al. 1997).

Certain fluorescent (optical) brighteners, such as diamino stilbene disulfonic acid derivative, significantly shorten time to death and increase larval mortality rates to baculoviruses (Shapiro 1992, Shapiro & Robertson 1992, Morales et al. 2001). These materials prevent sloughing of virus-infected midgut cells (Washburn et al. 1998), prevent apoptosis of virus-infected cells (Dougherty et al. 2006), or disrupt the peritrophic membrane in infected larvae (Wang & Granados 2000). These materials also protect viruses from deactivation by ultraviolet radiation (Martignoni & Iwai 1985, Shapiro & Dougherty 1994, Dougherty et al. 1996).

Insect cell lines are often used to evaluate or multiply insect viruses (Blissard 1996, Lynn 2002, 2003a, b, 2007). For example, in an attempt to develop *in vitro* techniques for production of AgMNPV, Castro et al. (1997) evaluated cell culture lines of four lepidopteran species. They found that infections of AgMNPV were highly productive only in cell cultures of *A. gemmatalis*. Production of AgMNPV was less efficient in cell lines of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), and unproductive in cell lines of two other species (Castro et al. 1997).

The overall objectives of this research were to identify entomopathogenic viruses that are active against pickleworm larvae and to investigate the use of fluorescent brighteners to enhance their activity against this pest.

## Materials and Methods

**Pickleworm colony.** Pickleworms used for bioassay studies were reared at the USDA-ARS, U.S. Vegetable Laboratory (USVL), Charleston, SC by methods modified from those of Elsey et al. (1984). Modifications included using a multi-purpose Lepidoptera diet (BioServe Product #F9772; Wheat Germ, Soy Flour Base) (BioServe, Frenchtown, NJ), the elimination of plastic louvers in the rearing crispers, and using layered instead of crumpled paper towels for pupation sites (Jackson et al. 1998). Larvae for bioassays were fed on the multi-purpose diet until the second instar.

**Virus isolates.** Twelve Nucleopolyheroviruses (Baculoviridae) were evaluated. They were: *Autographa californica* multiply-embedded NPV (AcMNPV) (Vail et al. 1972), *Anagrapha falcifera* multiply-embedded NPV (AnfaMNPV) (Hostetter & Puttler 1991), *Anticarsia gemmatalis* multiply-embedded NPV (AgMNPV) (Carner & Turnipseed 1977), *Galleria mellonella* multiply-embedded NPV (GmMNPV) (Fraser & Stairs 1982), *Helicoverpa armigera* singly-embedded

NPV (HearSNPV) (McCarthy et al. 1978, Hamm 1982), *Helicoverpa armigera* multiply-embedded NPV (HearMNPV) (Hamm 1982, Monroe & McCarthy 1984), *Helicoverpa zea* singly-embedded NPV (HzSNPV) (Ignoffo 1963, Chen et al. 2002), *Hyphantria cunea* multiply-embedded NPV (HycuMNPV) (Boucias & Nordin 1977), *Lymantria dispar* multiply-embedded NPV (LdMNPV) (Doane 1967), *Plutella xylostella* multiply-embedded NPV (PlxyMNPV) (Kariuki & McIntosh 1999), *Rachiplusia ou* multiply-embedded NPV (RoMNPV) (Paschke & Hamm 1961), and *Spodoptera exigua* multiply-embedded NPV (SeMNPV) (Smits & Vlak 1988). Two Granuloviruses (GV) (Baculoviridae) were evaluated: *Helicoverpa armigera* granulosis virus (HearGV) (Whitlock 1974) and *Pieris rapae* granulosis virus (PrGV) (David & Gardiner 1965). Three Cypoviruses (Cytoplasmic Polyhedrosis Viruses = CPV) (Reoviridae) were bioassayed: *Lymantria dispar* CPV (LdCPV) (Magnoler 1973), *Pectinophora gossypiella* CPV (PgCPV) (Ignoffo & Adams 1966), and *Trichoplusia ni* CPV (TnCPV) (Vail et al. 1969). Also, one Iridovirus (Iridoviridae) was evaluated: Invertebrate Iridescent Virus (IIV6) that was originally isolated from the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) (Fukaya & Nasu 1966).

The AcMNPV (strain 6-R) was obtained from E. M. Dougherty (USDA-ARS, Beltsville, Maryland). The AnfaMNPV was obtained from D. L. Hostetter (USDA-ARS, Kimberly, Idaho). The GmMNPV was obtained from G. R. Stairs (Ohio State University, Columbus, Ohio). The LdMNPV (LDP-226) was obtained from the U.S. Forest Service, Hampton, Connecticut. We originally obtained the PlxyMNPV from A. H. McIntosh (USDA-ARS, Columbia, MO). This isolate was produced in larvae of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Farrar et al. 2007). The HearSNPV (V-83 strain from China) was obtained from J. J. Hamm (USDA-ARS, Tifton, Georgia). We obtained a Brazilian isolate of AgMNPV from G. R. Carner (Clemson University, Clemson, SC). This isolate came from diseased larvae collected in southern Brazil (Carner & Turnipseed 1977). The AgMNPV was produced in laboratory-reared *A. gemmatalis* by standard techniques (Fuxa & Richter 1999). The RoMNPV was obtained from the virus collection at the Insect Biocontrol Laboratory (USDA-ARS, Beltsville, MD). The HycuMNPV was obtained from G. L. Nordin (University of Kentucky, Lexington, KY). The HearGV was obtained from J. J. Hamm (USDA-ARS, Tifton, GA). The PrGV was obtained from B. M. Shepard (Clemson University, Charleston, SC). The LdCPV was obtained from gypsy moth, *Lymantria dispar* (L.), larvae by the Asian Parasite Research Laboratory (USDA-ARS, Seoul, Korea) and was passed serially through gypsy moth larvae in our laboratory. The PgCPV was obtained from J. R. Adams (USDA-ARS, Beltsville, MD). The TnCPV was obtained from the virus collection at the Insect Biocontrol Laboratory (USDA-ARS, Beltsville, MD). The IIV6 was obtained from K. Hunter (USDA-ARS, Ft. Pierce, FL).

**Development of pickleworm cell lines.** The primary culture method and the initial procedure for cell line maintenance were essentially as previously described (Lynn 1996). *D. nitidalis* eggs on melon leaves were obtained from the rearing colony at the USVL in Charleston, SC. Leaf sections containing 3-d old eggs were submerged in 70% ethanol and the eggs gently teased off the leaf surface with forceps. The eggs were kept in alcohol for approximately 5 min, rinsed twice in 2 mL of sterile de-ionized water, and transferred to 0.2 mL of Ex-Cell™ 420 serum-free medium (SAFC Biosciences, Lenexa, KY) supplemented

with 5% fetal bovine serum (FBS) and 50 μg/mL gentamicin sulfate. Then, the egg chorions were crushed with microforceps to expel the embryos. Two-dozen embryos were transferred to 0.2 mL fresh medium in a standing drop in the center of a 35-mm tissue culture Petri dish (Falcon®, BD Biosciences, San Jose, CA) and cut with a microscapel into 4 to 8 pieces each. The Petri dish was sealed with Parafilm® (Pechiney Plastic Packaging Company, Menasha, WI) and incubated in a humidified plastic box at 26°C. After 24-h, an additional 1 mL medium was added to the culture. Additional 0.5 mL aliquots of medium were added at 7 to 14 d intervals. When the total volume in the culture reached approximately 3 mL, all but 0.5 mL of the medium was replaced with 0.5 mL fresh medium until the first sub-culture was made (8 weeks after initiation). Cells were gently flushed from the surface of the Petri dish and the medium transferred from the culture into a 25-$cm^2$ Greiner® tissue culture flask (Greiner Bio-One, Frickenhausen, Germany) with 3 mL fresh medium for the initial sub-culture. The medium was replaced at weekly intervals on this plate until the attached cells in the flask were nearly confluent. After 4 weeks, the attached cells were suspended by replacing the culture medium with Ca/Mg-free phosphate buffered saline that was then replaced with 50 μg/mL VMF-trypsin (virus- and mycoplasma-free). After 20 min at room temperature, 7 mL fresh medium was added to the culture and the cells suspended by gentle flushing of medium through a pipet. Half of the cell suspension was then transferred to a 25-$cm^2$ Greiner tissue culture flask and incubated at 26°C. Attached strains of cells in two primary cultures of *D. nitidalis* embryos (DnE) were subsequently sub-cultured at 1:2 split ratios at 1 to 2 week intervals and led to two strains designated IPLB-DnEa3 and IPLB-DnEa4. Cells in the spent medium of the first passage flasks were collected by low speed (50 × *g*) centrifugation and re-suspended in fresh medium to create two suspended strains (designated IPLB-DnEs1 and IPLB-DnEs2).

**Cell line maintenance.** Regular sub-cultures were performed on the strains after the third passage. Attached strains were initially split 1:2 on a weekly basis in 25-$cm^2$ Greiner tissue culture flasks using the procedure described by Lynn (2002) and Ex-Cell™ 420 supplemented with 5% FBS. After the cultures could be split on a regular weekly interval, no antibiotics were added to the medium. As the cells adapted to growth in the culture medium, the split ratios were increased, eventually reaching a consistent growth rate allowing a 1:15 weekly split.

The suspended strains were maintained by collecting medium from a mature (1- to 2-week old) culture while being careful to not dislodge attached cells. The medium (containing unattached cells) was centrifuged (50 × *g*, 10 min) and the pellet re-suspended in fresh medium and transferred to a tissue culture flask. As with the attached strains, cells were split 1:2 during the initial sub-cultures, but eventually could be split at 1:20 after several months in culture.

**Cell line characterization.** The Authentikit® system (Innovative Chemistry, Inc., Marshfield, MA) was used for characterizing the cell lines following the manufacturer's directions. Cell extracts were applied to 1% agarose gels, which were subjected to electrophoresis at 160 volts for 25 min. After that, the gels were stained for isocitrate dehydrogenase (ICD; Enzyme Commission [E.C.] code 1.1.1.42 [IUPAC-IUBMB Joint Commission on Biochemical Nomenclature 2008]), phosphoglucose isomerase (PGI; E.C. 5.3.1.9), malate dehydrogenase (oxaloace-

tate-decarboxylating) (ME; E.C. 1.1.1.40), or phosphoglucomutase (PGM; E.C. 5.4.2.2). The stains, buffers and gels were obtained from Innovative Chemistry, Inc. (Marshfield, MA). An extract from IPLB-Sf21cells (Vaughn et al. 1977) was also included on the gels for comparison. The isozyme migration patterns on these gels were also compared with gels of other cell lines maintained in our laboratory.

**Susceptibility of cell lines to viruses.** Twenty-four well plates (Greiner Bio-One, Frickenhausen, Germany) were initiated with cells at approximately half the cell density used in the normal passage in 0.5 mL medium plus 50 μg/mL gentamicin sulfate. Each well was then inoculated with 5 μL of one of the viruses listed in Table 1. In addition to setting up multiple wells with each of the new lines, some wells were set up with other continuous cell lines as positive controls for each tested virus. IPLB-Sf21AE cells (Vaughn et al. 1977) were used with AcMNPV, AnfaMNPV, GmMNPV, HearMNPV, PlxyMNPV, and RoMNPV; BCIRL-HzAM1 cells (McIntosh & Ignoffo 1983) were used with HzSNPV; IPLB-LdFB cells (Lynn et al. 1988) were used with LdMNPV; UFL-Ag286 cells (Sieburth & Maruniak 1988) were used with AgMNPV; and BCIRL-SeE CLG1 cells (Goodman et al. 2001) were used with SeMNPV. Plates were incubated at 22°C and observed periodically for cytopathology for up to 2 weeks post inoculation.

**Susceptibility of pickleworm larvae to viruses.** Bioassays of pickleworm larvae were modified from published techniques (Shapiro & Farrar 2003). Experiments were done in new 9-cm diam. plastic Petri dishes that were discarded after use. Suspensions of the virus isolates were pipetted onto the surface of the same multi-purpose diet used for colony rearing. The diet was about 0.6 cm thick. Sterile distilled water was pipetted onto the surface of the diet in the control treatments. Ten second-instar pickleworm larvae from the laboratory colony were gently placed on the surface of the diet using soft forceps.

In the first susceptibility experiment, nine viruses were evaluated. They were AcMNPV, AnfaMNPV, AgMNPV, GmMNPV, HearGV, HearSNPV, HycuMNPV, PlxyMNPV, and RoMNPV. All treatments were applied at $10^7$ viral occlusion

**Table 1. Viruses tested on pickleworm, *Diaphania nitidalis*, cell lines (technique described in Lynn and Ferkovich, 2004).**

| | Occlusion body | | |
|---|---|---|---|
| Virus treatment | Abbreviation | Group | Production |
| *Anagrapha falcifera* MNPV | AnfaMNPV | I | + |
| *Anticarsia gemmatalis* MNPV | AgMNPV | I | + |
| *Autographa californica* MNPV | AcMNPV | I | + |
| *Galleria mellonella* MNPV | GmMNPV | I | + |
| *Helicoverpa zea* SNPV | HzSNPV | II | − |
| *Helicoverpa armigera* SNPV | HearMNPV | II | + |
| *Lymantria dispar* MNPV | LdMNPV | II | − |
| *Plutella xylostella* MNPV | PlxyMNPV | I | + |
| *Rachiplusia ou* MNPV | RoMNPV | I | + |
| *Spodoptera exigua* MNPV | SeMNPV | II | − |

bodies (OB) per dish (9-cm diam. × 1.5 cm deep). One ml of virus suspension was pipetted onto the surface of the diet. For each replication, there were 5 plates with 10 larvae in each plate. An equal number of controls (untreated diet) were tested. This experiment was replicated three times. Thus, a total of 150 larvae were bioassayed for each treatment. Plates were examined several times over a two-week period, and the numbers of dead larvae, live larvae, prepupae, and pupae were recorded.

In the second susceptibility experiment, eight viruses were evaluated. They were AgMNPV, LdMNPV, HearGV, PrGV, LdCPV, PgCPV, TnCPV, and IIV6. The NPVs and CPVs were used at $10^7$ OBs per dish. The GVs and IIV6 were a 1/100 dilution of the stock suspension. The optical brightener, Blankophor P167 (Bayer Corp., Pittsburgh, PA, CAS #1670-24-9) at 1% (wt:wt) was tested alone and in combination with AgMNPV. For each replication, there were 10 plates with 10 larvae in each plate. This experiment was replicated two times. Thus, a total of 100 larvae were bioassayed for each treatment. Plates were examined several times over a two-week period, and the numbers of dead larvae, live larvae, prepupae, and pupae were recorded.

**Enhancement of viruses with brighteners.** In the first enhancement experiment, AgMNPV at $10^7$ OBs per dish was evaluated in combination with six optical (=fluorescent) brighteners. The fluorescent brighteners were Blankophor BBH (Bayer Corp., Pittsburgh, PA, CAS #4404-43-7), Blankophor HRS (Bayer; CAS #61951-69-7), Blankophor LPG (Bayer; CAS #133-66-4), Blankophor P167 (Bayer; CAS #1670-24-9), Blankophor RKH (Bayer; CAS #35632-99-6), and Tinopal UNPA-GX (Sigma-Aldrich Chemical Company, St. Louis, Mo.; CAS #4404-43-7). All materials were diluted in distilled water and were tested at a 1% concentration (wt:wt). These brighteners were selected for comparison, because they are all bistriazinyl derivatives of 4,4′-diamino-2,2′-disulfonic acid (Argauer & Shapiro 1997). Blankophors BBH, LPG, and RKH also contain formaldehyde polymers with sulfonated 1,19-oxybis(methylbenzene) sodium salts (Shapiro & Argauer 2001). For each replication, there were 5 plates with 10 larvae in each plate. This experiment was replicated two times. Thus, a total of 100 larvae were bioassayed for each treatment. Plates were examined several times over a two-week period, and the numbers of dead larvae, live larvae, prepupae, and pupae were recorded.

In the second enhancement experiment, AgMNPV at $10^7$ OB per dish was evaluated with and without the addition of Blankophor P167. Controls consisted of untreated diet and diet treated with Blankophor P167 alone. For each replication, there were 10 plates with 10 larvae in each plate. This experiment was replicated two times. Thus, a total of 200 larvae were bioassayed for each treatment. Plates were examined several times over a two-week period, and the numbers of dead larvae, live larvae, prepupae, and pupae were recorded.

In the third enhancement experiment, AnfaMNPV at $10^7$ OB per dish was evaluated with and without the addition of Blankophor P167. Controls consisted of untreated diet and diet treated with Blankophor P167 alone. For each replication, there were 10 plates with 10 larvae in each plate. This experiment was replicated three times. Thus, a total of 300 larvae were bioassayed for each treatment. Plates were examined several times over a two-week period, and the numbers of dead larvae, live larvae, prepupae, and pupae were recorded.

**Data analysis.** Data from larval bioassays were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of the SAS system, version 8.02 (SAS 1999). When treatment effects were significant at the 5% level, means were separated by Fisher's least significant difference (LSD) at the 5% probability level. (SAS 1999).

## Results and Discussion

**Cell lines.** Four cell line strains were isolated from *D. nitidalis* embryos two in suspension (IPLB-DnEs1 and IPLB-DnEs2) and two attached (IPLB-DnEa3 and IPLB-DnEa4). These cell lines are the first established from *D. nitidalis*. Although the strains were isolated from different primary cultures, few differences were observed between them with respect to morphology or in preliminary tests with viruses. Isozyme analysis showed identical patterns for all the new pickleworm cell lines, with migration distances of 10 mm for PGI, 12.5 mm for ME, and 13.5–16 mm for PGM. ICD was not detected in any of the pickleworm cell lines. The collective pattern of migration for these four enzymes was unique to the DnE lines when compared with other cell lines maintained in the USDA-ARS, Invasive Insect Biocontrol and Behavior Laboratory (formerly, the Beltsville Insect Biocontrol Laboratory) (see Lynn [2007] for available insect cell lines). Therefore, most of the results reported in this paper focus on one suspended cell line (IPLB-DnEs1) and one attached cell line (IPLB-DnEa3) from pickleworm embryos (Fig. 1).

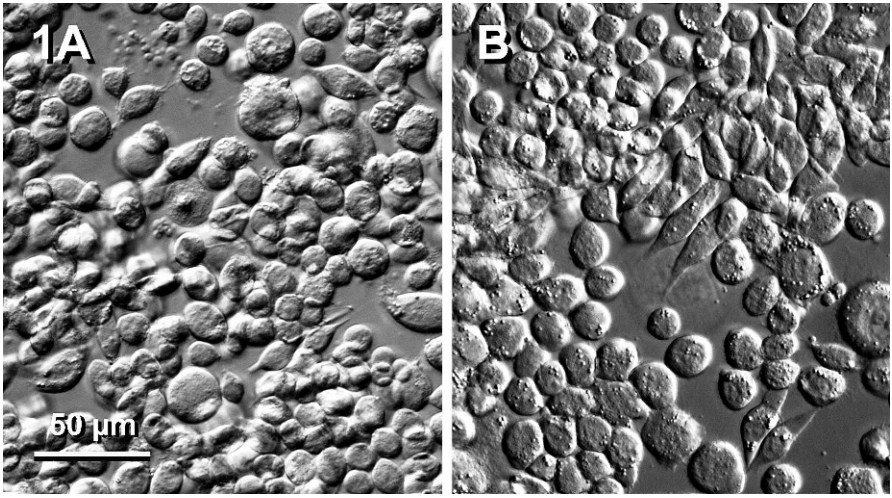


**Fig. 1.** *Diaphania nitidalis* embryonic cell lines. A. IPLB-DnEs1 cells at the 72nd passage; B. IPLB-DnEa3 cells at 66th passage. Both lines were photographed 8 d after the cultures were established using a Nikon Eclipse E400 inverted microscope with Differential Interference Contrast optics. Both figures are at the same magnification, marker bar is 50 µm.

**In vitro evaluation of viruses in pickleworm cell lines.** The pickleworm cell cultures were challenged with several NPVs (Table 1). The cell lines were fully susceptible to each of the Group I NPVs (AcMNPV, AgMNPV, AnfaMNPV, GmMNPV, PlxyMNPV and RoMNPV) (Zanotto et al. 1993, Bulach et al. 1999) with occlusion body production, but showed evidence of cytopathology to only one of the Group II viruses (HearMNPV) (Table 1). Relatively few individual cells (approximately 1 to 5%) showed OB production with most of the infectious virus strains (see RoMNPV-infected cells in Fig. 2A as an example), but most of the cells produced many OBs when inoculated with AgMNPV (Fig. 2B and 2C). Infection of the control permissive cell lines with each virus strain showed cytopathology with OB production, indicating the viral inocula were infectious. Screening of *D. nitidalis* cell line with 10 NPVs indicated AgMNPV was the most infectious of the tested viruses.

**Susceptibility of pickleworm larvae to viruses.** In the first experiment, ANOVA indicated that treatment effects (virus type) were highly significant ($F$ = 17.3, df = 9, 18, $P < 0.0001$). AgMNPV and AcMNPV killed significantly more pickleworm larvae than the other NPV treatments; however, the mortality rate was less than 30% (Table 2). RoMNPV, PlxyMNPV, and HearSNPV also killed significantly more larvae than the untreated control. Neither GmMNPV, HycuMNPV, nor HearGV were effective (Table 2). Interestingly, GmMNPV

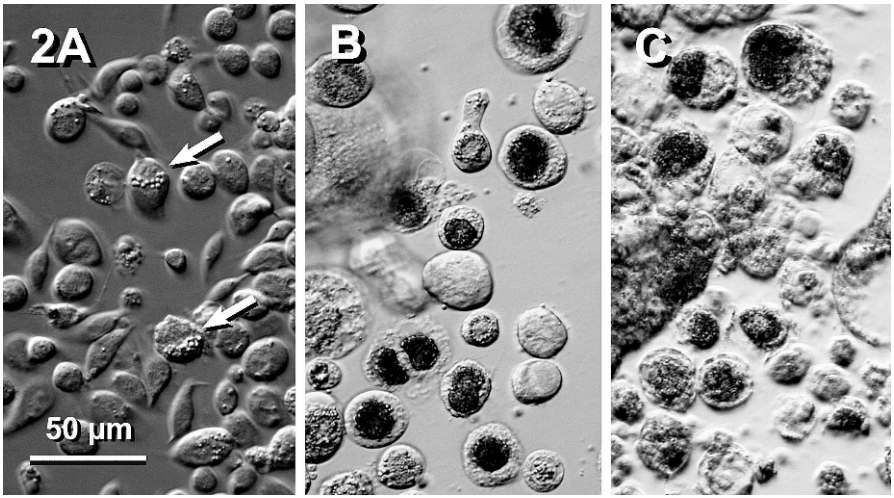


**Fig. 2.** *Diaphania nitidalis* cell lines after inoculation with viruses. A. DnE1s cells 8 d post-infection with RoMNPV. Arrows indicate cells with occlusion bodies in the nuclei; B. DnE1s cells 11 d post-infection with AgMNPV. The hypertrophic cells with dark nuclei are highly infected with many occlusion bodies; C. DnE3a cells 11 d post-infection with AgMNPV. The hypertrophic cells with dark nuclei are highly infected with many occlusion bodies. Cultures were photographed with a Nikon Eclipse E400 inverted microscope with Differential Interference Contrast optics. All figures are at the same magnification, marker bar is 50 µm.

produced occlusion bodies in the *D. nitidalis* cell line (Table 1), but this virus was not effective against pickleworm larvae *in vivo*.

In the second experiment, ANOVA indicated that treatment effects (virus type) were highly significant ($F = 185.5$, df = 11, 21, $P < 0.0001$). AgMNPV killed significantly more pickleworm larvae than any other virus treatment, and the mortality rate was similar to the first experiment (Table 3). None of the granuloviruses, cypoviruses, or the iridovirus killed any pickleworm larvae in this experiment. Similar to experiments 2 and 3, the addition of the fluorescent brightener, Blakophor P167 significantly improved mortality of second instar *D. nitidalis* to AgMNPV.

**Table 2. Effect of baculoviruses on mortality of second instar pickleworms in laboratory bioassays, Charleston, SC, 2007.**

| Virus treatment | Percent mortality |
|---|---|
| AcMNPV | 28.0 a |
| AgMNPV | 27.3 a |
| AnfaMNPV | 14.7 b |
| RoMNPV | 14.0 b |
| PlxyMNPV | 11.3 b |
| HearSNPV | 11.3 b |
| GmMNPV | 0.7 c |
| HycuNPV | 0.0 c |
| HearGV | 0.0 c |
| Untreated Control | 0.0 c |

Means followed by the same number are not significantly different according to Fisher's least significant difference (LSD) at the 5% probability level. (SAS 1999).

**Table 3. Effect of baculoviruses on mortality of second instar pickleworms in laboratory bioassays, Charleston, SC, 2007.**

| Virus treatment | Percent mortality |
|---|---|
| AgMNPV plus Blankophor P167 | 66.0 a |
| AgMNPV | 33.3 b |
| Blankophor Alone | 0.0 c |
| Control | 0.0 c |
| HearGV | 0.0 c |
| IIV6 | 0.0 c |
| LdCPV | 0.0 c |
| LdMNPV | 0.0 c |
| PgCPV | 0.0 c |
| PrGV | 0.0 c |
| TnCPV | 0.0 c |

Means followed by the same number are not significantly different according to Fisher's least significant difference (LSD) at the 5% probability level. (SAS 1999).

Although there has been one report of a nucleopolyhedrovirus from *Diaphania indica* in India (Narayanan & Veenakumari 2003), our study is the first report of nucleopolyhedroviruses affecting survival of pickleworm larvae, *D. nitidalis*. AcMNPV and AgMNPV caused significantly higher mortality in 2nd instar *D. nitidalis* than any of the other viruses. Interestingly, AnfaMNPV and RoMNPV, which are variants of AcMNPV (Blissard et al. 2000, Harrison & Bonning 1999, 2003), were less effective than AcMNPV.

**Enhancement of viruses with brighteners.** In the first experiment with fluorescent brighteners, ANOVA indicated that treatment effects (addition of a brightener) was highly significant ($F = 22.3$, df = 7, 8, $P < 0.0001$). The fluorescent brighteners Blankophor HRS, Blankophor BBH, Blankophor P167, Tinopal UNPA-GX, and Blankophor RKH significantly increased the mortality of AgMNPV against pickleworm larvae (Table 4). However, Blankophor LPG was ineffective, which is consistent with the study by Shapiro & Argauer (2001) who reported that Blankophor LPG was not effective in enhancing LdMNPV against gypsy moth or SeMNPV against the beet armyworm, *Spodoptera exigua*.

ANOVA indicated that treatment effects were highly significant in the second ($F = 73.6$, df = 3, 7, $P = 0.0038$) and third ($F = 804.84$, df = 3, 11, $P < 0.0001$) experiments. The addition of Blankophor P167 to AgMNPV or AnfaMNPV significantly increased susceptibility of 2nd instar *D. nitidalis* (Table 5). our results are consistent with previous studies that showed fluorescent brighteners increase the activity of AgMNPV against its velvetbean caterpillar host (Fuxa & Richter 1998, Morales et al. 2001).

In future studies, the promising viruses should be passed through pickleworm larvae or cell cultures to determine if the potency can be increased, as has been shown with other baculoviruses (Pavan et al. 1981). Subsequently, successful viruses need to be tested in replicated field experiments on cucurbit crops (small and large plot studies) (Shapiro & Farrar 2003, Shapiro et al. 2002). Field efficacy of viruses enhanced by optical brighteners, virus combinations, or other methods (Shapiro 2000a,b, Shapiro & Argauer 2001, Shapiro & Farrar 2003), also should

**Table 4. Effect of fluorescent brightners on efficacy of AgMNPV on mortality of second instar pickleworms in laboratory bioassays, Charleston, SC, 2007.**

| Virus treatment | Percent mortality |
|---|---|
| AgMNPV plus Blankophor P167 | 95.0 a |
| AgMNPV plus Tinopal UNPA-GX | 91.0 a |
| AgMNPV plus Blankophor BBH | 90.0 a |
| AgMNPV plus Blankophor RKH | 86.0 a |
| AgMNPV plus Blankophor HRS | 79.0 a |
| AgMNPV plus Blankophor LPG | 27.0 b |
| AgMNPV Alone | 21.0 b |
| Untreated Control | 0.0 c |

Means followed by the same number are not significantly different according to Fisher's least significant difference (LSD) at the 5% probability level. (SAS 1999).

**Table 5. Enhancement of two baculoviruses on mortality of second instar pickleworms in two laboratory bioassays, Charleston, SC, 2007.**

| Virus treatment | Percent mortality | |
|---|---|---|
| AgMNPV plus Blankophor P167 | 88.2 a | ---- |
| AgMNPV Alone | 29.7 b | ---- |
| AnfaMNPV plus Blankophor P167 | ---- | 69.3 a |
| AnfaMNPV Alone | ---- | 11.3 b |
| Blankophor P167 Alone | 0.0 c | 0.0 c |
| Untreated Control | 0.0 c | 0.0 c |

Means in the same column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) at the 5% probability level. (SAS 1999)

be tested against pickleworm larvae. Also, susceptibility at the cellular level will need to be verified.

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# Efficacy of Entomopathogenic Viruses on Pickleworm Larvae and Cell Lines[1]

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J. Agric. Urban Entomol. 25(2): 81–97 (April 2008)

**ABSTRACT** A critical need exists for new biological pest management techniques for the pickleworm, *Diaphania nitidalis* (Stoll) (Lepidoptera: Pyralidae), a major pest of Cucurbitaceae. One potential management strategy for this pest is the use of entomopathogenic viruses. In this study, several viruses were evaluated for efficacy against pickleworm cell lines and second-stage larvae. First, selected viruses were bioassayed *in vitro* against one suspended cell line (IPLB-DnEs1) and one attached cell line (IPLB-DnEa3) that we derived from pickleworm embryos. In these experiments, a multiply-embedded nucleopolyhedrovirus (AgMNPV) from velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, showed the greatest activity toward the pickleworm cell lines. Following these results, 14 entomopathogenic viruses in the families Baculoviridae, Reoviridae, and Iridoviridae were evaluated *in vivo* in bioassays of second instar *D. nitdalis*. Among these 14 viruses, only six baculoviruses showed significant activity against pickleworm larvae. AgMNPV and a multiply-embedded nucleopolyhedrovirus (AcMNPV) from alfalfa looper, *Autographa californica* (Speyer), were the most efficacious against second instar *D. nitidalis*. Moreover, five of six stilbene fluorescent brighteners significantly increased efficacy of AgMNPV against pickleworm larvae.

**KEY WORDS** baculovirus, AgMNPV, *Diaphania nitidalis*, Pyralidae, insect cell line

The pickleworm, *Diaphania nitidalis* (Stoll) (Lepidoptera: Pyralidae), is a serious pest of Cucurbitaceae (York 1992, Capinera 2001). Female pickleworm moths deposit eggs in small clusters on leaves and flower buds of cucumber, *Cucumis sativus* L.; cantaloupe, *Cucumis melo* L.; pumpkin, *Cucurbita pepo* L.; squash, *Cucurbita* spp.; and other cucurbits (Reid & Cuthbert 1956). Eggs hatch in 2–4 d, and young larvae initially feed close to where they hatch (Fulton 1947, Canerday & Dilbeck 1968). However, as larvae grow older they enter fruits, where they can cause severe economic damage (Fulton 1947). Control of the early instars is important because once the larvae penetrate the fruits, they are nearly impossible to kill with insecticides, and by then the fruits have been ruined and

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# Effect of Trap Color on Captures of Grape Root Borer (Lepidoptera: Sesiidae) Males and Non-Target Insects[1]

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**ABSTRACT** The effect of bucket trap color on grape root borer (GRB), *Vitacea polistiformis* Harris, captures was evaluated in 2005 and 2006. Traps were deployed in a commercial vineyard consisting of muscadine (*Vitis rotundifolia* Michx) grapes in North-Central Florida. Experimental designs were randomized complete block with four tratments including green, yellow, white and blue traps in 2005. In 2006, five treatments (green, yellow, green top, yellow top, and multicolor) and four replicates were evaluated. All traps were baited with GRB female sex pheromone. Trap color had a significant effect on the number of GRB males captured. In 2005, green and yellow traps caught more GRB males than other trap colors. In addition, first GRB male captures were in yellow and green traps. In 2006, the multicolor trap caught more GRB males than any other trap and had the first GRB capture. Grape root borer males prefer green and yellow pheromone-baited traps, but do not appear to distinguish between these two colors, which have similar spectral reflectance. Trap color had a significant effect on the number of Apoidea captured, with highest captures in multicolor traps. Choice of trap color can improve the effectiveness of pheromone-baited bucket traps for GRB in both early detection and total number captured over the season. Negative impacts on beneficial insects appear minimal.

**KEY WORDS** grape root borer, *Vitacea polistiformis*, monitoring, trapping, trap color, non-target

The principal types of grapes cultivated in Florida are Muscadines, *Vitis rotundifolia* Michx (Weihman 2005), which were initially thought to be resistant to grape root borer attack, but this has proven to be incorrect (Snow et al. 1991). The grape root borer (GRB), *Vitacea polistiformis* (Harris), is the most serious pest of cultivated grapes (*Vitis* spp.) in Florida (Liburd et al. 2004). The adults resemble paper wasps (*Polistes* spp.) and are distributed all along the eastern United States from Florida to New Jersey and west to Missouri and Arkansas. The northern limit of its geographic range appears to be on a line with central Pennsylvania and Ohio, but GRB adults have been trapped in southeastern Michigan (Snow et al. 1991).

In Florida, GRB adults emerge in the summer and are active for a period of four to six months (Webb et al. 1992, Weihman & Liburd 2007). Females produce a sex pheromone to attract mates and once mated deposit their eggs on weeds or grasses near the base of grapevines (Snow et al. 1991, Sarai 1972). One female

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can produce from 200 to 500 eggs (Clark & Enns 1964), and upon hatching, larvae immediately burrow into the soil in search of grape roots.

Grape root borer larvae feed within the cambium layer of the roots causing gouge-like wounds along the lateral roots and on the trunk base (Naegely 2001, Dutcher & All 1979). Damage to the roots not only affects water and mineral uptake, but also increases susceptibility to soil borne pathogens and reduces cold tolerance (Pearson & Meyer 1996). A single larva can have a significant effect on a grape vine, reducing yield by 50% (Williams et al. 2002). Two to three larvae are capable of killing an entire vine (Williams et al. 2002). In some areas, yield losses and vine death were so extensive that entire vineyards had to be replaced (Alm et al. 1989).

Grape root borer infestations are often difficult to identify. In addition, GRB spends most of its lifespan under the soil, and symptoms of vine decline may not appear until years after initial infestation (Harris et al. 1994). The period of vulnerability for GRB is relatively narrow; therefore, proper timing is critical to suppress populations. The use of pheromone-baited traps is important to monitor populations and determine whether or not GRB is present in a field. In addition, effective monitoring of GRB populations will allow growers to better time their sprays against adults and when first instar larvae are crawling into the soil. Once larvae enter the roots, they are sheltered from insecticides, predators, and other mortality factors (Harris et al. 1994).

Our goal is to improve monitoring tools for GRB. Weihman & Liburd (2007) previously showed that green bucket traps (Universal Moth Trap, Great Lakes IPM, Vestaburg, MI) were more effective than Delta-wing traps for capturing GRB. However, other colors of commercial bucket traps were not studied. The effect of color as a visual cue affecting trap performance has been studied for other lepidopteran pests (Athanassiou et al. 2004, Meagher 2001, Mitchell et al. 1989, Suckling et al. 2005). Moths for which studies have been done include fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Meagher 2001); velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Mitchell et al. 1989); jasmine moth, *Palpita unionalis* (Hübner) (Athanassiou et al. 2004); lightbrown apple moth, *Epiphyas postvittana* (Walker); codling moth, *Cydia pomonella* (L.) (Clare et al. 2000); and currant clearwing, *Synanthedon tipuliformis* (Clerck) (Suckling et al. 2005). The currant clearwing moth is the only other sesiid for which this type of research has been done. In most of the studies listed above, trap color did have a significant effect on the number of male moths captured.

The specific objectives of this study were 1) to determine the effect of trap color on the captures of GRB male moths in pheromone-baited bucket traps, and 2) to examine the effect of trap color on the number and diversity of non-target insects caught.

## Material and Methods

**Field site and bucket traps.** Experiments to determine the effect of trap color on GRB male captures were conducted at a commercial vineyard in Grandin, FL (Putnam County) in 2005 and 2006. This vineyard contained mixed grape varieties including 'Carlos', 'Welder', 'Higgins', 'Fry', 'Dixie Red', 'Sugargate', and 'Watergate'. Individual plots were 0.06 hectares (0.22 hectare per treatment). Rows were separated by approximately 3.0 m alleyways, and vines within each row were spaced 6.1 m apart. Bucket traps (Universal Moth Trap,

Great Lakes IPM, Vestaburg, MI) were hung from the trellis wire approximately 0.5 m from the main trunk of each vine at a height of 1.5 m. All traps were baited with 1 mg of GRB pheromone: 99% E,Z-2,13-octadecadien-1-ol acetate, 1% Z,Z-3,13-octadecadien-1-ol acetate (Great Lakes IPM, Vestaburg, MI). Each trap contained an insecticide strip (Hercon Vaportape II, Hercon Environmental, Emigsville, PA) to kill captured insects.

*2005.* The experiment was designed as a randomized complete block (blocked by variety) with four replicates. Four bucket trap treatments (trap color) were evaluated: 1) green, 2) yellow, 3) white (Krylon® 1502 Flat White, Krylon Products Group, Cleveland, OH) and 4) blue (Krylon® 1910 True Blue Gloss). Green and yellow traps were obtained from a commercial source (Great Lakes IPM, Vestaburg, MI). White and blue traps were made by spray painting green bucket traps. Each trap was spaced approximately 20 m apart. Trap position was rotated weekly. Traps were initially placed in the field on 8 July and the number of GRB males captured was recorded each week through 1 November. Pheromone lures were replaced (after six weeks) on 23 August.

*2006.* Based on the 2005 results, the treatments were modified in 2006 to investigate various color combinations of bucket traps. Five treatments (trap color combinations) were evaluated that included: 1) multicolor bucket trap (green lid, yellow funnel, white bucket), 2) yellow top bucket trap (yellow lid, yellow funnel, green bucket), 3) green top bucket trap (green lid, green funnel, yellow bucket) (Fig. 1. A, B, and C), 4) all green, and 5) all yellow bucket traps. The experimental design was randomized complete block with four replicates. Similar to 2005, traps were spaced 20 m apart within the same vineyard. Traps were set out in the field on 31 July (three weeks later than the previous year because monitoring data from 2006 indicated a later flight for GRB). Traps were rotated weekly within each replicate. Pheromone lures were replaced (after 10 weeks) on 16 October. The number of GRB males and non-target insects was recorded each week through 6 November. Non-target insects were removed from the buckets and placed in re-sealable plastic bags. These insects were brought to the Small Fruit and Vegetable IPM Laboratory at the University of Florida and identified to family level using keys (Triplehorn & Johnson 2005).

**Statistical analysis.** The number of males captured per week was compared across trap colors using repeated measures analysis of variance (ANOVA), and differences among means were determined using a least significant difference (LSD) mean separation test ($\alpha = 0.05$) (PROC MIXED, SAS Institute 2003). Data were log10(x+1) transformed to satisfy model assumptions. Untransformed means and standard errors are reported in the tables and figures.

Mean numbers of non-target insects were compared across trap colors for each order using a one-way analysis of variance and log10(x+1) transformation. Means for non-target insects were separated using Tukey's Studentized Range (HSD) test ($\alpha = 0.05$) (SAS Institute 2003). The number of hymenopterans was analyzed based on superfamily, most falling within Apoidea and Vespoidea. Differences between superfamily means were analyzed using a *t*-test.

## Results

*2005.* Overall, trap color had a significant effect on the number of GRB males captured (Fig. 2). Green and yellow bucket traps caught significantly more male

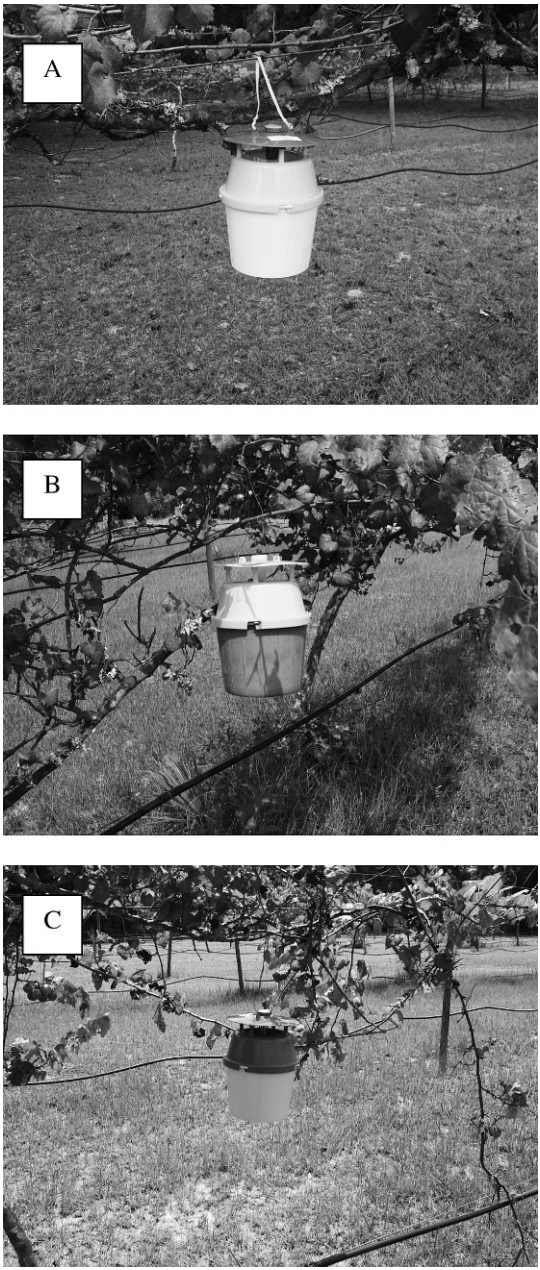


**Fig. 1.** Bucket traps with multiple color components (lid, funnel, base) used in 2006. A) Multicolor (green, yellow, white); B) Yellow Top (yellow, yellow, green); C) Green Top (green, green, yellow).

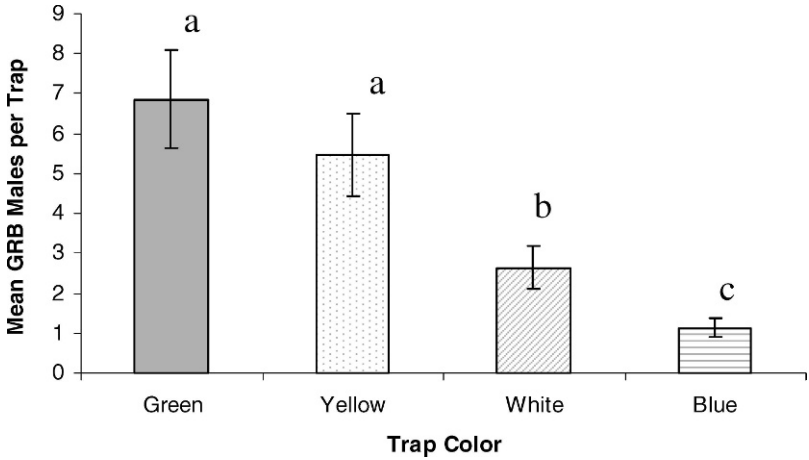


**Fig. 2.** Grape root borer males caught in each color trap in 2005. Bars with the same letter are not significantly different at the 0.05 level (LSD).

moths than white or blue traps ($F$ = 30.38; df = 3,153; $P < 0.0001$). Green and yellow traps captured 2.6 and 2.1 times as many male moths as white traps and 6.1 and 4.8 times as many male moths as blue traps, respectively (Fig. 2). The interaction effects between treatment and time were significant ($F$ = 1.67; df = 48,153; $P$ = 0.010) in 7 out of the 17 weeks sampled (Table 1). The green, yellow and white traps all showed a similar pattern of captures increasing at the end of August and decreasing from the beginning of October onward (Fig. 3). Captures of male GRB peaked around 20 September. For the blue bucket traps, the number of males captured slowly increased from the beginning of September to their peak at 4 October. The last grape root borer males were collected on 1 November. First capture was recorded in yellow bucket traps, and first multiple captures were recorded in green bucket traps. The overall distribution of trap captures over the season was unimodal.

*2006.* Similarly in 2006, trap color had a significant effect on the number of male GRB caught (Fig. 4). Multicolor bucket traps caught significantly more GRB males than any other trap color when averaged over the entire season ($F$ = 8.89; df = 4,168; $P < 0.0001$). Also, first capture was recorded in multicolor bucket traps. There were no significant differences among trap captures for all green, all yellow, green top or yellow top traps. The interaction effects between treatment and time were not significantly different ($F$ = 1.11; df = 52,168; $P$ = 0.300).

Grape root borer males were caught the first week of sampling, 7 August, and numbers began to increase immediately, peaking on 25 September (Fig. 5). Apart from all green bucket traps, there were no well-defined peaks for any other trap color as in 2005. All yellow and yellow top traps (Fig. 1B), however, showed a bimodal distribution of trap captures over the season. The multicolor trap captured 1.7 times as many male GRB as the other traps evaluated.

Various adult insects from 65 families were collected. For many of these families only a few individuals were captured. Most non-target individuals captured were Hymenoptera, followed by Diptera, Lepidoptera, Coleoptera, and Hemiptera (Table 2). Traps caught high numbers of lovebugs, *Plecia nearctica* Hardy (Diptera:

**Table 1. Weekly mean trap captures (± SEM) in different colored bucket traps over the 2005 sampling period. Only weeks in which significant differences were observed are reported.**

| Trap Color | 5-Aug | 19-Aug | 23-Aug | 30-Aug | 20-Sep | 27-Sep | 11-Oct |
|---|---|---|---|---|---|---|---|
| Green | 0.75 (0.25) a | 1.00 (0.41) ab | 1.50 (0.29) a | 3.25 (1.03) a | 25.5 (6.36) a | 18.8 (6.96) a | 12.8 (3.17) a |
| Yellow | 0.0 (0.0) b | 2.25 (0.25) a | 1.25 (0.75) a | 3.75 (1.32) a | 13.3 (5.36) a | 15.0 (4.44) a | 9.50 (1.71) ab |
| White | 0.50 (0.29) ab | 0.50 (0.50) b | 0.75 (0.25) ab | 2.75 (0.85) a | 10.3 (2.93) ab | 8.50 (3.07) ab | 4.25 (1.65) bc |
| Blue | 0.0 (0.0) b | 0.25 (0.25) b | 0.0 (0.0) b | 0.0 (0.0) b | 3.00 (1.08) b | 3.00 (1.41) b | 1.75 (0.85) c |

Means followed by the same letter are not significantly different at the 0.05 level (LSD).

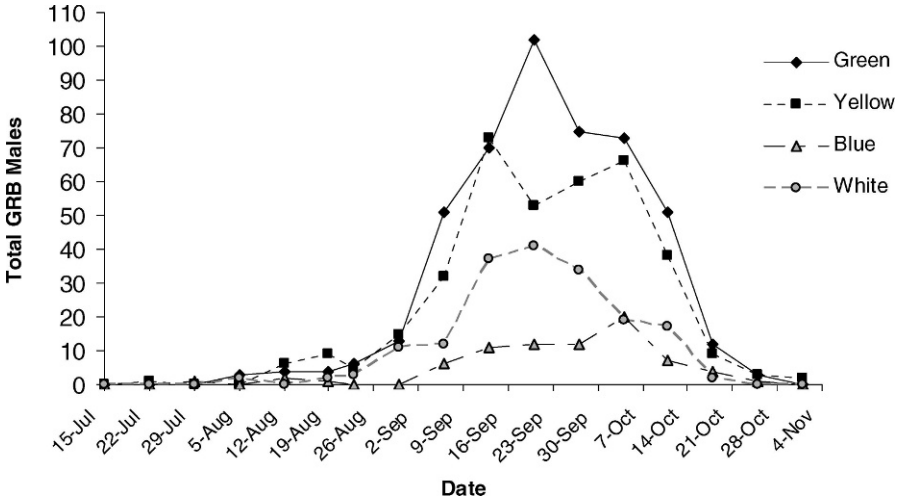


**Fig. 3.** Seasonal pattern of trap captures of grape root borer males in different colored pheromone-baited bucket traps in 2005.

Bibionidae), in mid- to late September. This family was not included in Table 1 in order to avoid obscuring the differences among other groups. The multicolor trap was the only trap that caught significantly more Diptera than the all green trap. None of the other treatments were different. All treatments captured significantly more Vespoidea than Apoidea ($t = 5.94$; df = 70.3; $P < 0.0001$), but there was no statistical difference in the number of Vespoidea among trap colors (Fig. 6). In Apoidea, the multicolor trap caught significantly more than the all green, green top,

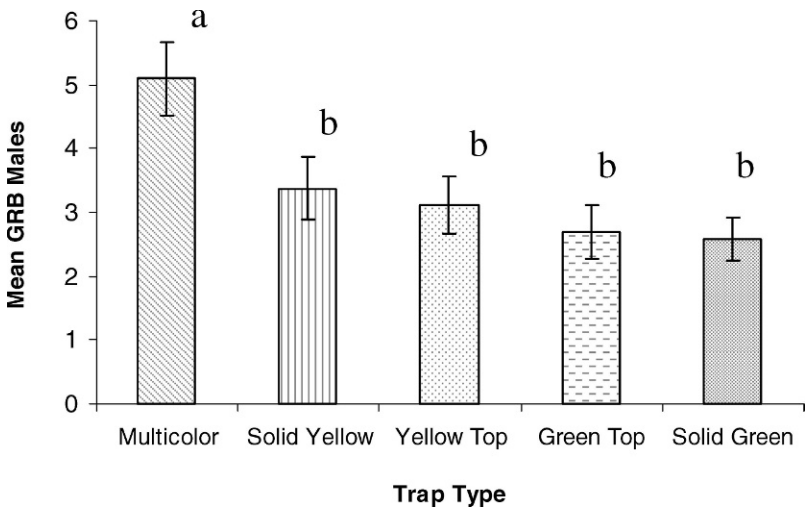


**Fig. 4.** Grape root borer males caught in each trap color in 2006. Bars with the same letter are not significantly different at the 0.05 level (LSD).

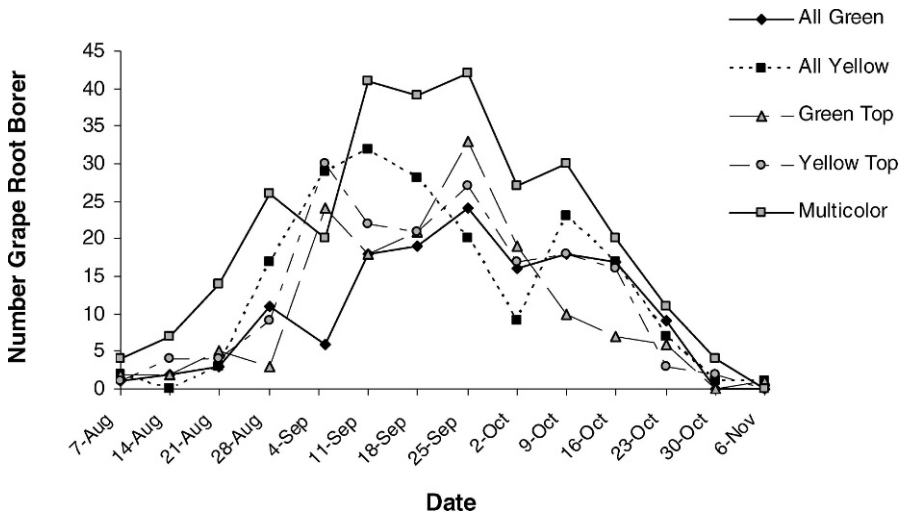


**Fig. 5.** Seasonal pattern of trap captures of grape root borer males in different colored pheromone-baited bucket traps in 2006.

and yellow top traps ($F$ = 2.71; df = 4,65; $P$ = 0.038). The four most commonly captured families were Formicidae, Vespidae, Tiphiidae, and Apidae. Very few bees in the family Apidae were captured.

## Discussion

Grape root borer, as evidenced by the 2005 data, can distinguish between yellow or green and white bucket traps. While white bucket traps alone were not attractive, the combination of colors with contrasting reflectance seems to make the traps more noticeable and attract more moths. Mitchell et al. (1989) found similar results for velvetbean caterpillar and fall armyworm. Both these species, however, are nocturnal. Meagher (2001) also found the multicolor trap to be most effective for fall armyworm. Unlike GRB, the all green traps did not capture large

**Table 2. Weekly mean trap captures (± SEM) of non-target insects in different colored bucket traps baited with grape root borer sex pheromone.**

| | Orders | | | | |
|---|---|---|---|---|---|
| Trap | Coleoptera | Diptera* | Hemiptera | Hymenoptera | Lepidoptera |
| Multicolor | 0.93 (0.22) | 3.07 (0.72) a | 0.643 (0.31) | 5.86 (1.61) | 2.79 (0.74) |
| All Yellow | 0.64 (0.34) | 2.07 (0.66) ab | 1.214 (0.56) | 7.07 (2.55) | 1.21 (0.40) |
| Green Top | 1.21 (0.35) | 1.57 (0.54) ab | 0.214 (0.11) | 4.71 (1.14) | 1.29 (0.50) |
| Yellow Top | 1.36 (0.34) | 2.07 (0.62) ab | 0.714 (0.32) | 2.43 (0.79) | 1.00 (0.39) |
| All Green | 1.36 (0.33) | 0.50 (0.23) b | 0.214 (0.11) | 3.07 (1.02) | 0.86 (0.28) |

*Means followed by the same letter are not significantly different at the 0.05 level (Tukey's).

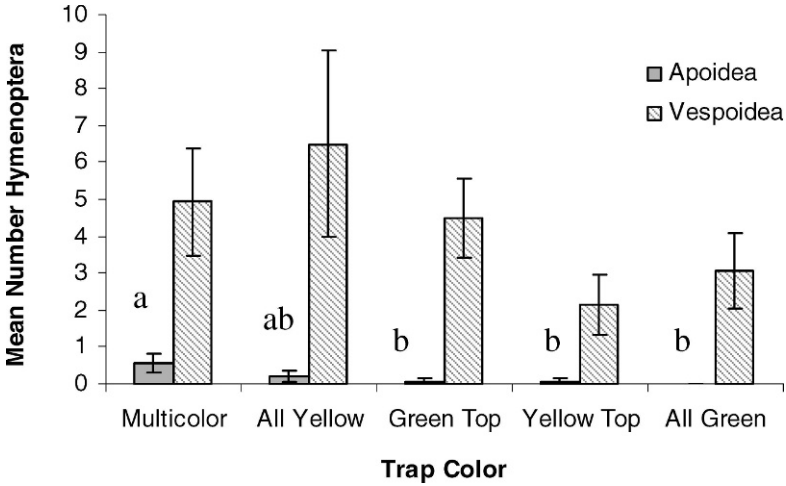


**Fig. 6.** Mean number of Apoidea and Vespoidea (±SEM) captured in different colored bucket traps per week in 2006. In Apoidea, bars with the same letter are not significantly different at the 0.05 level (LSD).

numbers of nocturnal moths. This may be explained by low light reflectance which would make the green trap less noticeable at night.

For effective monitoring, a trap should combine early detection and high counts over the sampling period. In both 2005 and 2006, the trap colors which captured the most GRB males were also the first to capture males. These traps were effective for detecting the presence of GRB at high and low population densities.

Color can be used to enhance the effectiveness of pheromone-baited traps. Differences in attractiveness depend on the spectral reflectance of each given color. According to Suckling et al. (2005), green and yellow traps have similar spectral reflectance. This explains why there were no significant differences in trap captures between green and yellow traps in 2005, and among the traps with different combinations of green and yellow parts (all green, all yellow, green top, and yellow top) in 2006. Suckling et al. (2005) found no significant differences between green and yellow delta traps for currant clearwing moths, and blue and white delta traps were not effective. It is not surprising that GRB and currant clearwing have similar responses to trap color; both are day-flying sesiid moths. The attractiveness of the multicolor trap (green lid, yellow funnel, white bucket) over the all green and yellow traps seems counterintuitive since the all white trap was ineffective.

The seasonal patterns for 2005 and 2006 agree with trapping data from other studies done in North-Central Florida (Webb et al. 1992, Weihman & Liburd 2007). The distribution of trap captures for 2005 in this study closely mirrored the Alachua County results from Webb et al. (1992). Although Snow et al. (1991) state that there is a tendency toward bimodal peaks of male flight activity in the south, this was not supported by this study. The difference in trap captures from 2005 to 2006 shows that GRB flight activity can vary considerably from one year to the next. Factors affecting adult emergence and flight activity are not well known but may involve climate and host plant phenology (Webb et al. 1992). Weihman & Liburd (2007) also attribute differences in seasonal distribution to

changes in weather conditions. Clearly, a better understanding of the physiological effects of climate on GRB development and the interaction of host plant phenology with emergence are required.

The primary concerns of non-target captures in monitoring devices are the loss of beneficials, such as pollinators, and reduced trap efficacy. Previous research on the capture of non-target Hymenoptera in traps focused on *Bombus* spp., *Apis mellifera*, and native bee species (Clare et al. 2000, Meagher & Mitchell 1999, Meagher 2001). Very few bees were captured in 2006. The numbers of Apoidea captured was comparable with data from Meagher (2001) and Meagher & Mitchell (1999). The Hymenoptera totals were greatly influenced by the large number of ants (Formicidae) captured. Ants were common on the grape vines as were aphids. The presence of ants in the traps was probably influenced more by the distribution of aphids than trap color. Although few bees were captured and the number of Hymenoptera captured overall was low, the multicolor traps did catch significantly more Apoidea than the traps with green parts. Adams et al. (1989) report catching more stinging Hymenoptera in yellow and white traps, and suggest using all green traps to reduce non-target captures. Whether or not the difference is enough to impact pollinator populations has yet to be determined. Spiders (Aranae) and insect orders in which only a small number ($<5$) of individuals were collected, such as Neuroptera and Orthoptera, were not included in the analysis.

Non-target species can increase the time needed to service traps and complicate pest identification if the non-target insects are closely related (Adams et al. 1989). In the current study, pest identification was not complicated by non-target captures. Only a few non-target sesiids were captured over the season. Identification of GRB was complicated in cases where specimens were degraded by rainwater collected in the buckets, or consumed by small vertebrates, such as tree frogs. Trap servicing time was affected by high numbers of lovebugs (Diptera: Bibionidae) captured in mid- to late September. In this study, traps were checked once per week so lovebugs were able to accumulate over seven days. More frequent servicing of traps would alleviate this problem.

Trap efficacy, target specificity, and early detection are important factors in developing monitoring strategies. Pheromone-baited bucket traps can accurately detect the flight period of GRB adults, and trap efficacy can be improved by choice of trap color. Further research is recommended for a better understanding of the effect of trap color on the diversity and abundance of non-target insects captured.

## Acknowledgments

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# Effect of Trap Color on Captures of Grape Root Borer (Lepidoptera: Sesiidae) Males and Non-Target Insects[1]

Craig R. Roubos and Oscar E. Liburd

J. Agric. Urban Entomol. 25(2): 99–109 (April 2008)

**ABSTRACT** The effect of bucket trap color on grape root borer (GRB), *Vitacea polistiformis* Harris, captures was evaluated in 2005 and 2006. Traps were deployed in a commercial vineyard consisting of muscadine (*Vitis rotundifolia* Michx) grapes in North-Central Florida. Experimental designs were randomized complete block with four tratments including green, yellow, white and blue traps in 2005. In 2006, five treatments (green, yellow, green top, yellow top, and multicolor) and four replicates were evaluated. All traps were baited with GRB female sex pheromone. Trap color had a significant effect on the number of GRB males captured. In 2005, green and yellow traps caught more GRB males than other trap colors. In addition, first GRB male captures were in yellow and green traps. In 2006, the multicolor trap caught more GRB males than any other trap and had the first GRB capture. Grape root borer males prefer green and yellow pheromone-baited traps, but do not appear to distinguish between these two colors, which have similar spectral reflectance. Trap color had a significant effect on the number of Apoidea captured, with highest captures in multicolor traps. Choice of trap color can improve the effectiveness of pheromone-baited bucket traps for GRB in both early detection and total number captured over the season. Negative impacts on beneficial insects appear minimal.

**KEY WORDS** grape root borer, *Vitacea polistiformis*, monitoring, trapping, trap color, non-target

The principal types of grapes cultivated in Florida are Muscadines, *Vitis rotundifolia* Michx (Weihman 2005), which were initially thought to be resistant to grape root borer attack, but this has proven to be incorrect (Snow et al. 1991). The grape root borer (GRB), *Vitacea polistiformis* (Harris), is the most serious pest of cultivated grapes (*Vitis* spp.) in Florida (Liburd et al. 2004). The adults resemble paper wasps (*Polistes* spp.) and are distributed all along the eastern United States from Florida to New Jersey and west to Missouri and Arkansas. The northern limit of its geographic range appears to be on a line with central Pennsylvania and Ohio, but GRB adults have been trapped in southeastern Michigan (Snow et al. 1991).

In Florida, GRB adults emerge in the summer and are active for a period of four to six months (Webb et al. 1992, Weihman & Liburd 2007). Females produce a sex pheromone to attract mates and once mated deposit their eggs on weeds or grasses near the base of grapevines (Snow et al. 1991, Sarai 1972). One female

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# Comparative Susceptibility of Adult and Larval Lesser Mealworms, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), Collected from Broiler Houses in Arkansas to Selected Insecticides[1]

C. Dayton Steelman

J. Agric. Urban Entomol. 25(2): 111–125 (April 2008)

**ABSTRACT** Loss of insecticide susceptibility was suspected in lesser mealworms, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), in broiler chicken facilities in Arkansas due to reports of control failures. Bioassay tests using selected insecticides were conducted on adult and larval beetles collected from broiler chicken production farms having different insecticide application history. The residual and topical application tests showed that previous insecticide use had caused significant differences in the relative susceptibility of lesser mealworms. Topical application bioassay tests indicated that adult beetles had greater susceptibility to the insecticides tested than 8th instars collected from the same farm while in residual tests little difference was generally found in the susceptibility of adults and larvae exposed to the insecticides. Data suggested that the ability to de-toxify DDT and other organochlorine insecticides remained in some beetle populations due to spread of wood-shaving litter containing beetles over pastures among broiler production facilities, particularly involving facilities that have been in broiler production for over 40 y. A program utilizing integrated tactics that will reduce the dependency on insecticides is needed to manage litter beetles in poultry production facilities.

**KEY WORDS** litter beetles, lesser mealworm, insecticide susceptibility, *Alphitobius diaperinus*

The number of poultry production facilities being treated with insecticides to manage the lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), continues to increase in response to the poultry industries attempts to prevent significant economic production losses attributed to the presence of the beetles. Initially, the beetle infestations were reported as problematic when the late instars migrated from the wood-shaving or rice hull litter up the facility walls and tunneled into the insulation (Collison 1980) in search of a site in which to molt to the pupal stage followed with the newly emerged adults tunneling out of the insulation. This resulted in extensive economic damage to the insulation (Despins et al. 1987) and resulted in increased heating and cooling costs. Second, starting with the broiler flock initiation with 1-d-old chicks the broilers eat the larval and adult beetles, thus, consuming any

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pathogens carried on or in the beetles. They have been reported to be competent reservoirs of *Salmonella typhimurium* (McAllister et al. 1994), *Escherichia coli* (McAllister et al. 1996), *Campylobacter jejuni* (Jacobs-Reitsma et al. (1995, Strother et al. 2005), Infectious Bursal Disease Virus (McAllister et al. (1995) and other pathogens (De las Casa et al. 1973, De las Casa et al. 1973, 1976). Third, the feed conversion and weight gain of broilers and turkey poults have been shown to decrease due to the consumption of the adult and larval beetles (Despins & Axtell 1995). Fourth, the adult beetles become pests in human residences and recreation facilities after the litter has been removed and distributed in forage areas adjacent to human habitation (Gall 1980) due to their attraction to light.

Although many insecticides have been used to control the beetles, no base line data relative to the susceptibility to any insecticides was reported for any population until Vaughan and Turner (1984) collected and tested beetles from a deep pit commercial egg facility in Virginia. At this facility permethrin had been sprayed 3–4 times each week and cyromazine had been used as a feed-through for fly control. They reported the results of bioassay tests using both topical application and residual tests on polystyrene and unpainted plywood. Due to the increased use of insecticides, loss of field efficacy is repeatedly reported in Arkansas as well as in other states. Field efficacy loss to Iodofenphos SC, fenitrothion WP, and permethrin and resistance to Iodofenphos and malathion has been reported in the United Kingdom (Wakefield and Cogan 1991, Cogan et al. 1996), as well as cyfluthrin in southern Australia (Lambkin 2005, Lambkin and Rice 2006).

Here we report the comparative susceptibility of field populations of adult and larval beetles collected from commercial broiler houses that had previously been treated with different insecticides over different periods of time. In addition, the impact of pasture applications of wood-shaving litter that contains all stages of the beetle on resistance development as these beetles mix into other beetle populations is reported.

## Materials and Methods

Adult and larval litter beetles were collected from broiler houses at four locations. Farms were chosen to represent beetle populations that had been exposed to different chemical classes of insecticides. Using Fayetteville, AR, as a central point, Farm A was located approximately 30 km east of Fayetteville, AR, and had been in broiler production for over 40 y during which time the broiler facilities had been treated with organochlorine, organophosphate, carbamate and pyrethriod insecticides. Farm B was located approximately 30 km west southwest of Fayetteville, AR, and had been in production for 12 y. The beetles collected from this farm were used as a susceptible population for comparison with beetles from other farms because there was no previous history of insecticide use. In addition, Farm B was located in an area where agricultural land had been used primarily for apple production and was >20 km from other poultry farms. Farm C was located approximately 35 km north northwest of Fayetteville, AR and had been in production for 6 y. The beetle population from this farm had been exposed to successive treatments with permethrin and cyfluthrin. Farm D was located ≈5 km west of Farm A or ≈25 km east of Fayetteville. This farm was included in the study because although the farm had been in broiler production

for 2 y, only the pyrethroid cyfluthrin had been applied at the initiation of each flock grow-out and had resulted in unsatisfactory beetle control. Litter that contained all life stages of the beetles from Farm A had been distributed on pastures immediately adjacent to the broiler houses at Farm D, during spring clean-outs for 40 y, and including both years since broiler production was initiated at Farm D.

Beetles were collected from two broiler houses at each location. Wood-shaving litter that contained larval and adult beetles was collected from beneath feeder pans in each house using a metal cylinder that had a 550.6 cc dry volume capacity and placed in 4-liter plastic buckets. Two, 4-liter plastic buckets that contained wood-shaving litter and adult and larval beetles collected from beneath the feeder pans were filled from three areas of the house (16.6 m from each end and in the center of the house). Thus, 6 buckets containing beetles were transported to the laboratory and combined in equal parts into 50 cm wide × 55 cm long × 20 cm deep wooden boxes that were covered with a Plexiglas plate. Five boxes containing the beetles from each house were maintained in temperature and humidity control rearing cabinets (Revco, The Netherlands) at 27 ± 2°C, 60% RH, and a photoperiod of 16:8 (L:D)h. The beetles were fed commercial starter chicken feed that contained corn and soybean meal (Chick Starter, Herider Farms, Fayetteville, AR) that contained vitamin and mineral supplements. The beetles were provided water weekly by placing a 2-$cm^2$ piece of water-saturated sponge on the litter surface. The beetles from each farm were maintained in separate rearing cabinets.

In preparation for either topical application or residual bioassay tests, approximately 500, 6th instars (7 mm in length, according to Wilson and Minor 1969), were removed from the field collections and placed in 27.5 long × 17.5 wide × 17.5 cm deep covered plastic chambers (Penn Plax Large Animal Carrier, Garden City, NY) that were maintained at 27 ± 2°C, 60% RH, and a photoperiod of 16:8 (L:D)h. The beetles were fed commercial starter chicken feed and water weekly as previously described. When cast skins indicated that the larvae had molted twice and reached the 8th instar (8 mm in length (Wilson and Miner 1969), they were removed and used as a uniform age group for the bioassay tests. Other 8th instars were maintained in the chambers that were lined with corrugated paper (Northwest Arkansas Paper Company, Springdale, AR) and additional 30 $cm^2$ rolled into a cylinder for pupation shelter until newly emerged adults were available for testing. The newly emerged adults were removed and held in Petri dishes containing 1 g finely ground chicken feed and a 1 cm square piece of water soaked sponge in groups of 100 for 5 d, at which time they had completely tanned (Hopkins et al. 1992) and were used for the bioassay tests.

*Topical application bioassay tests.* Bioassay tests were performed by topical application of a 0.5 μl drop that contained a specific concentration of insecticide (in acetone) to the prosternum of each larval and adult beetle using a Hamilton micro-applicator (Hamilton Co., Reno, NV). Treatment concentrations in micrograms per gram (μg/g) were calculated using the mean weight (n = 100 of each stage) of 8th instar (15.01 ± 1.66 μg) and adult (14.27 ± 1.58 μg) beetles. Adult and larval beetles were individually removed from the untreated Petri dishes previously described, and after treatment, each beetle was placed in an untreated individual 4 cm tall shell vial to allow drying. After 1 h the beetles were removed from the vials and combined into disposable Petri dishes and held

for 24 and 48-h observations. Untreated control beetles were treated with a 0.5 μl drop of acetone and handled as described for the beetles treated with various insecticide concentrations.

Preliminary tests were conducted with 4 dilution series concentrations of each insecticide to determine the appropriate concentration range for the adult and larval beetles from each location. The preliminary concentrations for DDT and methoxychlor were 1000, 500, 100, and 50 μg/g, for cyfluthrin, cypermethrin, tetrachlorvinphos, and chlorpyrifos the series dilutions were 10, 1.0, 0.1 and 0.01 μg/g, and for carbaryl and permethrin 100, 10, 1.0 and 0,1 μg/g concentrations were used. After the initial tests, 5 to 10 series dilution concentrations of each insecticide were used that would bracket the concentrations used in the preliminary tests providing a range of 0–100% mortality as well as an untreated control. The concentration/mortality tests were replicated four times and these data were used to determine the susceptibility of the beetles. Twenty-five adult or larval beetles were used in each replication. Mortality counts were taken at 24 and 48 h after exposure. Beetles that did not move when touched with an insect pin were scored as dead.

*Residual bioassay tests.* The insecticide concentrations were applied to glass petri dishes by dispensing 1 ml of the appropriate concentration (insecticide and acetone mixture) into the bottom portion of each Petri dish following the procedures of Plapp (1971). The dishes were then rotated by hand so that the liquid coated the entire bottom of the dish and the acetone evaporated. Untreated control Petri dishes were prepared by dispensing 1 ml of acetone into the bottom portion of the dishes and rotating the dish by hand so that the acetone coated the entire bottom of the dish. After acetone evaporation the top portion of the dish was placed on the bottom and sealed with masking tape until used for testing.

Prior to conducting the residual tests, 25 adult or 8th instars were placed in the bottom portion of sterile disposable (14 cm diameter and 2 cm high) Petri dishes, covered with the top portion and held at 21 ± 2°C until ready for testing. When ready for testing, the 25 larval or adult beetles were poured into the treated bottom portion of the Petri dishes, covered with the top portion and held at 21 ± 2°C. After 1 h exposure, the beetles were poured back into the untreated disposable Petri dishes and mortality counts taken at 24 and 48 h post exposure. Death was defined as a lack of any movement when prodded with an insect pin.

Adult and larval litter beetles were tested against the chlorinated hydrocarbons DDT (98% [AI], Sigma, St. Louis, MO) and methoxychlor (96.2% [AI], Kincaid Enterprises, Nitro, W. VA): organophosphates, chlorpyrifos (Dursban, Dow-Elanco, Indianapolis, IN), tetrachlorvinphos (Rabon, Fermenta Animal Health, Kansas City, MO); carbamate, carbaryl (Sevin, Rhone-Poulenc, Courbevoie Cedex, France); and pyrethroids, cyfluthrin (Tempo, Bayer, Corporation, Kansas City, MO), cypermethrin (92% [AI], Zeneca Ag. Products, Wilmington, DE), and permethrin (Ectiban 95.3% [AI], FMC, Princton, NJ).

The data were analyzed with probit analysis (SAS Institute 1985). Resistance ratios were calculated by dividing the $LC_{50}$ of the appropriate susceptible population into the $LC_{50}$ of each field population tested. $LC_{50}$'s from each lesser mealworm population were compared and considered to be significantly different from the susceptible population when their 95% fiducial limits did not overlap those of the susceptible population.

## Results and Discussion

**History of previous insecticide use.** Records of previous insecticide applications that were directed at several insect pests over many years provide important information that was used to interpret base-line susceptibility data obtained on the beetles. The lesser mealworms obtained from commercial broiler farms in the present study had been exposed to insecticide treatments for varying numbers of years. At each farm, no beetle monitoring system to evaluate insecticide treatment efficacy had been used. Acceptable beetle control had been generally considered acceptable if after applications, few live adults or larvae were observed during the 1st and 2nd wk following treatment even if beetle numbers generally increased rapidly by the 5th and 6th wk of each grow-out. Concern developed about insecticide efficacy when in continuous flock grow-outs adult and larval numbers remained high during the 1st and 2nd wk of each flock grow-out after treatment, and increased significantly with each succeeding grow-out where insecticides were applied between each flock grow-out.

*Farm A.* Farm A had been in broiler production for >40 y and during the 10-yr-period that extended from the mid-1950s to the mid-1960s, DDT and Lindane were recommended (Barnes et al. 1954, 1958, 1968, 1970, 1974, Jones and Johnson 1972, 1973, 1979, 1982, 1988) and applied intermittently to the wood-shaving litter for the control of a variety of poultry pests that included the litter beetle. Because of the previous history of use by broiler producers and the similarity of insecticide-resistance mechanisms between chlorinated hydrocarbons and pyrethroids (Scott 1990), DDT and methoxychlor were included in the bioassay tests to determine if loss in susceptibility could be detected due to the previous use of these insecticides.

After the chlorinated hydrocarbons were no longer registered, several registered organophosphates (malathion, coumaphos, naled, and dichlorvos) were applied to the wood-shaving litter in the broiler houses on this farm (Barnes et al. 1954, 1958, 1968, 1970, 1974, Jones and Johnson 1972, 1973, 1979, 1982, 1988) beginning in the mid-1960s. In the 1970s, tetrachlorvinphos was applied as a spray to the litter prior to the initiation of each flock grow-out and in the 1980s, a tetrachlorvinphos-dichlorvos mixture was used prior to the start of the 3rd, 4th and 5th flock grow-outs each year. In the late 1980s, the pyrethroid, permethrin, was found to provide longer beetle control during the last weeks of the 3rd, 4th and 5th flock grow-outs than the tetrachlorvinphos-dichlorvos mixture. The carbamate, carbaryl, was registered and generally applied as a spray to the wood-shaving litter prior to the 1st flock grow-out on the farm beginning in the 1960s (Jones and Johnson 1972, 1973, 1979, 1982, 1988). However, due to unsatisfactory beetle control with this insecticide especially in the 3rd, 4th and 5th flock grow-outs each year it was generally used only prior to the 1st grow-out.

*Farm B.* Farm B had been in broiler production for 12 y, beginning in the late 1990s and no pesticides had been used at this farm. Although it is possible that lesser mealworms that had been previously exposed to insecticides could have infested these facilities preliminary baseline susceptibility tests indicated that they were susceptible to the label concentrations of both tetrachlorvinphos and cyfluthrin.

*Farm C.* Broiler production started at Farm C in the late-1990s. Permethrin was used exclusively during the 1st year of production between each of the 5 flock

grow-outs for beetle control but cyfluthrin was used between each of the 5 flock grow-outs beginning at the start of the 2nd y and was the only insecticide used during the next 5 y. No organophosphate or carbamate insecticides had been used to manage the beetle population at Farm C.

*Farm D.* This farm had been in broiler production for 2 y and only one pyrethroid (cyfluthrin), supplied by the integrator poultry company, had been used for *A. diaperinus* control. Cyfluthrin had been applied to the litter of these facilities prior to the start of 10 flock grow-outs and had not provided satisfactory reduction in beetle numbers. Litter removed from Farm A that contained all life stages of the beetles had been distributed on pastures between Farm A and the location of the Farm D broiler houses for >40 y and continued during the time of this study.

**Bioassays Tests.** Many of the insecticides that are currently registered for controlling the lesser mealworm act on contact with terrestrial life stages and resistance monitoring techniques have been primarily based on data obtained using topical application or residue (treated surface) contact methods of exposure (ffrench-Constant & Roush 1990). Mortality data from topical application bioassay tests quantifies the amount of a toxicant that has been received by each individual insect but this method does not reflect the mode by which residual insecticides are acquired by the beetles in actual control applications (Hinkel et al. 1985). However, residual bioassay tests provide data that represents exposure of the beetles to a dry residue of the insecticide on a substrate that closely simulates field applications. The combined data from these two bioassay methods provides useful information for determining the reasons for beetle control failures.

*Chlorinated hydrocarbons.* Due to field reports of loss of field effectiveness to pyrethroids, DDT was included in the materials tested because of suspicion of kdr-type nerve insensitivity involved in the resistance. Several mechanisms can cause resistance to DDT (Oppenoorth 1985) and DDT often yields better results than pyrethroids in identifying resistance because it generally provides higher resistance levels (Sawicki 1978, Scott et al. 1986) and it can be used to detect resistance to several compounds.

Topical application - Topical application bioassay data indicated that adult beetles from Farms A and D were significantly less susceptible to DDT than beetles from Farms B and C, where no chlorinated hydrocarbons had been used (Table 1). The beetle concentration-mortality data from Farm B was used as a susceptible population to determine the resistance ratio (RR) for beetles from Farms A and D. Using the $LC_{50}$ concentrations, the adult beetles had RR values of 3.7 and 2.8 and the larvae 3.4 and 2.5 for Farms A and D, respectively, to DDT. No significant difference existed in the susceptibility of the adult or larval beetles from Farms A and D. These data suggested that the beetles removed with the litter from Farm A and distributed in the pasture areas between Farms A and D became established in the broiler houses at Farm D when broiler production was initiated, thus, spreading the beetle's resistance to chlorinated hydrocarbons to the new broiler facilities.

The chlorinated hydrocarbon, methoxychlor, had not been used at Farm A, thus, the beetles from Farm A were compared to the beetles from Farm C that had only been exposed to pyrethroids. Adult beetles from Farm A were significantly less susceptible to methoxychlor than the beetles tested from Farm

C, however, no significant difference existed between larval susceptibility. The concentration of methoxychlor necessary to cause 50% mortality to both larvae and adults was high from Farms A and C and similar to the values found for DDT, although methoxychlor had never been applied to the broiler facilities at either farm. Although no chlorinated hydrocarbons had been used at Farm A for over 25 y, the adult and larval beetles at both Farms C and B were significantly more susceptible to DDT than the beetles from Farms A and D.

Residual tests - The susceptibility of adult and larval lesser mealworms collected at Farms A, B and C and exposed to selected insecticides in residual bioassay tests is shown in Table 2. The residue bioassay tests showed that the larval beetles were significantly more resistant to DDT than larvae from Farm B where no insecticides had ever been applied. The Resistance Ratio (RR) was 5.1 and 3.5 between the larval beetles from Farms A and B, respectively, suggesting that a high proportion of the larval population had the ability to detoxify and survive a treatment of this insecticide. Farm C had been in broiler production for 6 y and although no chlorinated hydrocarbons had been used at Farm C, the pyrethriods, permethrin and cyfluthrin, had been used to control the beetle population between each flock grow-out (5 flock grow-outs each year) during the 6-yr-period. The low level of susceptibility of the adults and larvae at Farm A where DDT and lindane had been applied followed by extensive use of the pyrethroids permethrin and cyfluthrin was highly suggestive of a target site kdr-like resistance mechanism. The kdr-type of insecticide resistance is exemplified by cross-resistance to DDT and its analogs and to pyrethroids (Sawicki 1978, Scott et al. 1986). In addition, no significant difference was found between the susceptibility of the adult or larval beetles from Farms A and C, to DDT or methoxychlor (Table 2).

*Pyrethroids.* After many years of extensive use, field reports indicated that permethrin was failing to provide adequate field control as determined by the presence of large numbers of live adults and larvae present in the wood shaving litter 1–2 week after treatment.

Topical application - The adults and larvae from Farm A were significantly less susceptible to topical exposure to permethrin (>14 times) when compared to the $LC_{50}$ data obtained from beetles collected at Farm B where no insecticides had been used during the 12 y of broiler production (Farm A and B adults $LD_{50}$ = 1.358 and 0.094 μg/g and larvae $LD_{50}$ = 94.31 and 6.61 μg/g, respectively). The adults and larvae at Farm A had a 14 RR, respectively, for permethrin when compared to the susceptible population at Farm B (Table 1) and although permethrin had been used for 5 successive flock grow-outs during the 1st year of production at Farm C, the adults and larvae were significantly more susceptible ($LD_{50}$ = 0.143 and 10.27 μg/g, respectively 0, than the beetles from Farm A. However, the Farm C beetles were significantly less susceptible to permethrin than the beetles from Farm B (Table 1).

The susceptibility of larval and adult beetles from Farm B to cyfluthrin was not significantly different from beetles tested from Farm C where cyfluthrin had been used for 5 y. However, they were significantly more susceptible than the beetles from Farms A and D. Although cyfluthrin had been applied between each of the 5 flock grow-outs during the 2 y of broiler production at Farm D the larvae were significantly more susceptible than those tested from Farm A. The concentration/response data of the Farm A and D beetles for cyfluthrin was

**Table 1. Susceptibility of field-collected adult and larval lesser mealworms to topically applied selected insecticides.**

| Location[a] | Stage | Number | Slope +/− SE | LC50 (95% Fiducial limits)[b] | RR[c] | $X^2$ | df |
|---|---|---|---|---|---|---|---|
| | | | **Cyfluthrin** | | | | |
| Farm C | Adult | 1000 | 2.56+/−0.49 | 1.466 (0.637–2.186) a | - | 0.9 | 3 |
| Farm B | Adult | 500 | 2.22+/−0.19 | 1.833 (1.607–2.082) b | - | 2.5 | 3 |
| Farm D | Adult | 2100 | 1.71+/−0.15 | 5.052 (4.176–6.372) b | 2.8 | 2.3 | 3 |
| Farm A | Adult | 500 | 1.93+/−0.58 | 6.846 (4.862–14.524) b | 3.7 | 3.5 | 3 |
| Farm C | Larvae | 1000 | 1.17+/−0.29 | 105.46 (45.84–157.28) a | - | 0.9 | 3 |
| Farm B | Larvae | 500 | 2.22+/−0.19 | 129.1 (113.15–146.60) a | - | 1.0 | 3 |
| Farm D | Larvae | 525 | 2.15+/−0.29 | 331.99 (260.92–437.97) b | 2.5 | 2.8 | 3 |
| Farm A | Larvae | 500 | 1.93+/−0.58 | 450.41 (319.88–955.50) b | 3.4 | 2.9 | 3 |
| | | | **Methoxychlor** | | | | |
| Farm C | Adult | 500 | 2.11+/−0.19 | 1.568 (1.340–1.881) a | - | 2.6 | 3 |
| Farm A | Adult | 750 | 1.43+/−0.33 | 8.04 (4.360–21.508) b | | 5.3 | 3 |
| Farm C | Larvae | 500 | 1.32+/−0.19 | 367.69 (284.3–545.43) a | - | 1.7 | 3 |
| Farm A | Larvae | 525 | 1.45+/−0.18 | 489.75 (398.9–645.4) a | | 1.6 | 3 |
| | | | **Cyfluthrin** | | | | |
| Farm B | Adult | 500 | 1.59+/−0.19 | 0.07 (0.054–0.085) a | - | 2.5 | 3 |
| Farm C | Adult | 500 | 1.41+/−0.18 | 0.079 (0.064–0.085) a | 1.1 | 0.5 | 3 |
| Farm D | Adult | 500 | 1.84+/−0.19 | 0.101 (0.086–0.117) b | 1.4 | 2.7 | 3 |
| Farm A | Adult | 2175 | 1.86+/−0.17 | 0.173 (0.109–0.228) b | 2.5 | 0.5 | 3 |
| Farm B | Larvae | 500 | 1.59+/−0.19 | 5.05 (3.89–6.17) a | - | 0.5 | 3 |
| Farm C | Larvae | 500 | 1.41+/−0.18 | 5.22 (4.22–6.22) a | 1.0 | 1.0 | 3 |
| Farm D | Larvae | 500 | 1.84+/−0.19 | 7.11 (6.3–8.26) b | 1.4 | 0.1 | 3 |
| Farm A | Larvae | 2175 | 1.86+/− 0.17 | 12.04 (9.65–15.81) c | 2.4 | 1.4 | 3 |
| | | | **Cypermethrin** | | | | |
| Farm B | Adult | 500 | 1.70+/−0.20 | 0.066 (0.052–0.080a | - | 1.8 | 3 |
| Farm C | Adult | 500 | 1.42+/−0.18 | 0.077 (0.061–0.194) a | 1.2 | 2.7 | 3 |
| Farm A | Adult | 750 | 1.34+/−0.24 | 0.087 (0.042–0.196) a | 1.3 | 0.9 | 3 |
| Farm C | Larvae | 500 | 1.42+/−0.18 | 5.57 (4.38–6.76) | - | 1.0 | 3 |
| | | | **Permethrin** | | | | |
| Farm B | Adult | 500 | 2.02+/−0.20 | 0.094 (0.081–0.0108) a | - | 2.8 | 3 |
| Farm C | Adult | 500 | 1.44+/−0.17 | 0.143 (0.119–0.175) b | 1.5 | 5.3 | 3 |
| Farm A | Adult | 700 | 0.93+/−0.11 | 1.358 (0.878–2.217) c | 14.4 | 1.6 | 3 |
| Farm B | Larvae | 500 | 2.02+/−0.20 | 6.61 (5.67–7.60) a | - | 2.5 | 3 |
| Farm C | Larvae | 500 | 1.44+/−0.17 | 10.27 (8.53–12.57) b | 1.6 | 0.9 | 3 |
| Farm A | Larvae | 700 | 0.93+/−0.11 | 94.31 (60.26–153.99) c | 14.3 | 2.5 | 3 |
| | | | **Carbaryl** | | | | |
| Farm C | Adult | 500 | 2.55+/−0.22 | 0.304 (0.264–0.361) a | - | 2.3 | 3 |
| Farm D | Adult | 900 | 1.22+/−0.09 | 0.316 (0.324–0.474) a | - | 0.1 | 3 |
| Farm B | Adult | 900 | 1.22+/−0.09 | 0.444 (0.380–0.556) a | - | 1.8 | 3 |
| Farm A | Adult | 700 | 2.05+/−0.46 | 8.451 (6.49–14.23 ) b | 19.0 | 2.7 | 3 |
| Farm C | Larvae | 500 | 2.55+/−0.22 | 21.89 (18.99–25.9) a | - | 5.1 | 3 |
| Farm D | Larvae | 900 | 1.22+/−0.09 | 22.27 (22.9–33.4) ab | 1.0 | 2.3 | 3 |
| Farm B | Larvae | 900 | 1.22+/−0.09 | 31.52 (26.41–38.6) b | - | 1.4 | 3 |
| Farm A | Larvae | 700 | 2.05+/−0.46 | 55.97 (42.37–93.4) c | 2.6 | 1.4 | 3 |

**Table 1. Continued.**

| Location[a] | Stage | Number | Slope +/− SE | LC50 (95% Fiducial limits)[b] | RR[c] | $X^2$ | df |
|---|---|---|---|---|---|---|---|
| | | | **Tetrachlorvinphos** | | | | |
| Farm B | Adult | 500 | 1.95+/−0.22 | 0.059 (0.045–0.067) a | - | 0.5 | 3 |
| Farm D | Adult | 600 | 2.21+/−0.22 | 0.085 (0.074–0.097) b | 1.4 | 2.9 | 3 |
| Farm C | Adult | 500 | 1.56+/−0.26 | 0.153 (0.099–0.270) c | 2.6 | 0.9 | 3 |
| Farm A | Adult | 750 | 2.27+/−0.025 | 0.219 (0.178–0.278) c | 3.7 | 1.0 | 3 |
| Farm B | Larvae | 500 | 1.95+/−0.22 | 4.01 (3.26–4.81) a | - | 0.5 | 3 |
| Farm D | Larvae | 600 | 2.21+/−0.22 | 5.98 (5.19–6.80) b | 1.5 | 2.8 | 3 |
| Farm A | Larvae | 1800 | 2.20+/−0.15 | 9.89 (8.08–12.39) c | 2.5 | 1.0 | 3 |
| Farm C | Larvae | 500 | 1.56+/−0.26 | 10.08 (6.49–17.77) c | 2.5 | 2.5 | 3 |
| | | | **Chlorpyrifos** | | | | |
| Farm C | Adult | 500 | 1.60+/−0.18 | 0.09 (0.075–0.107) a | - | 0.5 | 3 |
| Farm B | Adult | 500 | 2.42+/−0.19 | 0.098 (0.086–0.115) ab | | 1.4 | 3 |
| Farm D | Adult | 500 | 2.40+/−0.20 | 0.119 (0.115–0.134) b | 1.2 | 0.4 | 3 |
| Farm A | Larvae | 500 | 2.40+/−0.20 | 8.36 (7.41–9.46) | - | 1.6 | 3 |

[a]Broiler Farms: Farm A, located 30 km east of Fayetteville, AR (beetles exposed to organochlorine, organophosphate, carbamate and pyrethroid class insecticides for over 40 y; Farm B, located 30 km west southwest of Fayetteville (susceptible population, no known insecticide use for 12 y); Farm C, located 35 km north northwest of Fayetteville (beetles exposed to pyrethroids insecticides), and Farm D, located 5 km west of Farm A (beetles exposed only to pyrethroids). $LC_{50}$ values within the same stage and insecticide not followed by the same letter are significantly different based on overlap of 95% fiducial limits ($P < 0.05$).

[b]Expressed in μg/g.

[c]Resistance Ratios (RR) calculated by dividing the $LC_{50}$ of the appropriate susceptible population into the $LC_{50}$ of each field population tested.

similar to our bioassay data obtained for DDT relative to the movement of wood shaving litter containing beetles from one area to another as a part the facility clean-out and litter distribution process. Lambkin (2005) reported the loss of fenitrothion field effectiveness and later loss of cyfluthrin (Lambkin and Rice 2006) as well. Loss of permethrin field effectiveness had occurred at both Farm A and D and the dosage-mortality data indicated the need for additional data for decision making relative to a change away from the use of pyrethroid insecticides.

The concentration/mortality data indicated that although the beetles appeared to have developed resistance to the pyrethroid permethrin, the $LC_{50}$ values indicated that the beetles were more susceptible to cyfluthrin than permethrin. Roush et al. (1986) suggested that the best insecticide for resistance monitoring may be one that has not been used in the field. They reported that another pyrethroid, cypermethrin, appeared to been more effective to monitor pyrethroid resistance in the horn fly, *Haematobia irritans* L., than permethrin, thus, this pyrethroid was added to the bioassay tests. Cypermethrin had not been registered for or used to control *A. diaperinus* or other pests in broiler production. The results of the topical application tests showed that no significant difference ($P > 0.05$) existed between the susceptibility of the adult beetles at Farms A, B, or C to cypermethrin and comparison of the adult beetle $LC_{50}$ values indicated that the beetles from these farms were more susceptible to cypermethrin than cyfluthrin.

**Table 2. Susceptibility of field-collected adult and larval lesser mealworms exposed to residual treatments of selected insecticides.**

| Location[a] | Stage | Number | Slope +/−SE | $LC_{50}$ (95% FL)[b] | RR[c] | $X^2$ | df |
|---|---|---|---|---|---|---|---|
| | | | **DDT** | | | | |
| Farm C | Adult | 500 | 1.43+/− 0.14 | 0.86 (0.71–1.059) a | - | 6.9 | 3 |
| Farm A | Adult | 1750 | 1.31+/− 0.20 | 0.973 (0.654–2.73) a | - | 0.5 | 3 |
| Farm B | Larvae | 500 | 2.24+/− 0.3 | 0.082 (0.017–0.31) a | - | 3.4 | 3 |
| Farm C | Larvae | 500 | 1.41+/−0.4 | 0.286 (0.185–0.447) b | 3.5 | 3.3 | 3 |
| Farm A | Larvae | 500 | 1.61+/−0.9 | 0.421 (0.421–1.63) b | 5.1 | 1.3 | 3 |
| | | | **Methoxychlor** | | | | |
| Farm C | Adult | 1750 | 2.01+/−0.3 | 0.971 (0.577–3.41) a | - | 0.5 | 3 |
| Farm A | Adult | 500 | 2.11+/−0.19 | 1.568 (1.34–1.88) a | - | 2.5 | 3 |
| Farm C | Larvae | 600 | 2.15+/−0.1 | 0.671 (0.585–0.773) a | - | 1.6 | 3 |
| Farm A | Larvae | 500 | 1.92+/−0.29 | 1.112 (0.732–3.13) a | - | 1.8 | 3 |
| | | | **Cyfluthrin** | | | | |
| Farm B | Adult | 500 | 1.95+/−0.25 | 0.03 (0.021–0.04) a | - | 2.5 | 3 |
| Farm C | Adult | 500 | 2.01+/−0.16 | 0.063 (0.055–0.073) b | 2.1 | 0.8 | 3 |
| Farm A | Adult | 500 | 2.70+/−0.20 | 0.07 (0.062–0.079) b | 2.3 | 1.6 | 3 |
| Farm B | Larvae | 500 | 1.63+/−0.5 | 0.035 (0.029–0.049) a | - | 1.8 | 3 |
| Farm C | Larvae | 500 | 3.11+/−0.2 | 0.067 (0.059–0.107) b | 1.9 | 2. 5 | 3 |
| Farm A | Larvae | 500 | 2.62+/−0.27 | 0.183 (0.062–0.312) b | 5.2 | 0.8 | 3 |
| | | | **Cypermethrin** | | | | |
| Farm B | Adult | 500 | 2.59+/−0.31 | 0.064 (0.05+/–0.081) a | - | 3.5 | 3 |
| Farm C | Adult | 500 | 2.96+/−0.23 | 0.098 (0.082+/–0.103) b | 1.5 | 0.4 | 3 |
| Farm A | Adult | 900 | 2.73+/−0.1 | 0.179 (0.087–0.973) b | 2.8 | 2.6 | 3 |
| Farm B | Larvae | 500 | 1.14+/−0.32 | 0.091 (0.082–0.211) a | - | 0.7 | 3 |
| Farm A | Larvae | 500 | 3.13+/−0.2 | 0.068 (0.053–0.077) b | - | 2.1 | 3 |
| | | | **Permethrin** | | | | |
| Farm B | Adult | 500 | 2.7+/−0.42 | 0.073 (0.054–0.079) a | - | 0.5 | 3 |
| Farm C | Adult | 500 | 2.6+/−0.2 | 0.085 (0.08–0.186) b | 1.2 | 1.3 | 3 |
| Farm A | Adult | 500 | 1.91+/−0.15 | 0.092 (0.084–0.231) b | 1.3 | 2.5 | 3 |
| Farm C | Larvae | 500 | 2.0+/−0.2 | 0.091 (0.066–0.131) a | - | 1.5 | 3 |
| Farm A | Larvae | 500 | 1.71+/−0.31 | 0.232 (0.208–0.421) b | - | 3.3 | 3 |
| | | | **Carbaryl** | | | | |
| Farm B | Adult | 500 | 1.71+/−0.5 | 0.1417 (0.12–0.257) a | - | 0.5 | 3 |
| Farm C | Adult | 500 | 2.66+/−0.156 | 0.156 (0.127–0.21) a | 1.1 | 3 | |
| Farm A | Adult | 500 | 1.92+/−0.15 | 0.303 (0.26–0.353) b | 3.3 | 3.4 | 3 |
| | | | **Chlorpyrifos** | | | | |
| Farm A | Adult | 1000 | 3.95+/−0.38 | 0.081 (0.071–0.093) a | - | 1.3 | 3 |
| Farm C | Adult | 500 | 1.56+/−0.26 | 0.153 (0.099–0.27) b | 1.6 | 4.3 | 3 |
| Farm B | Adult | 1250 | 3.21+/−0.1 | 0.097 (0.037–1.17) ab | - | 1.2 | 3 |
| Farm B | Larvae | 500 | 1.6+/−0.2 | 0.071 (0.042–0.173) a | - | 0.6 | 3 |
| | | | **Tetrachlorvinphos** | | | | |
| Farm C | Larvae | 500 | 1.71+/−0.2 | 0.112 (0.086–0.27)a | 1.6 | 1.1 | 3 |
| Farm A | Larvae | 500 | 2.44+/−0.13 | 0.135 (0.118–0.156) a | 1.9 | 0.4 | 3 |
| Farm C | Adult | 1000 | 2.94+/−0.23 | 0.042 (0.039–0.050) a | - | 5.3 | 3 |

**Table 2. Continued.**

| Location[a] | Stage | Number | Slope +/−SE | $LC_{50}$ (95% FL)[b] | RR[c] | $X^2$ | df |
|---|---|---|---|---|---|---|---|
| Farm B | Adult | 500 | 2.44+/−0.28 | 0.073 (0.057–0.93) b | - | 4.1 | 3 |
| Farm A | Adult | 900 | 1.61+/−0.2 | 0.372 (0.101–0.477) c | 5.1 | 1.2 | 3 |
| Farm C | Larvae | 500 | 3.01+/−0.4 | 0.088 (0.057–0.188) a | - | 0.8 | 3 |
| Farm A | Larvae | 500 | 2.85+/−0.5 | 0.271 (0.192–0.216) b | - | 1.1 | 3 |

[a]Broiler Farms: Farm A, located 30 km east of Fayetteville, AR (beetles exposed to organochlorine, organophosphate, carbamate and pyrethroid class insecticides for over 40 y; Farm B, located 30 km west southwest of Fayetteville (susceptible population, no known insecticide use for 12 y); Farm C, located 35 km north northwest of Fayetteville (beetles exposed to pyrethroids insecticides), and Farm D, located 5 km west of Farm A (beetles exposed only to pyrethroids). $LC_{50}$ values within the same stage and insecticide not followed by the same letter are significantly different based on overlap of 95% fiducial limits ($P < 0.05$).

[b]Expressed in μg/g.

[c]Resistance Ratios (RR) calculated by dividing the $LC_{50}$ of the appropriate susceptible population into the $LC_{50}$ of each field population tested.

Comparison of Arkansas $LD_{50}$ topical application bioassay data with data published by Vaughan & Turner 1984) showed that the adult beetles tested in Arkansas were more susceptible to permethrin (Farm B, 0.094, Farm C, 0.143 and Farm A, 1.358 μg/g) than the adult beetles tested in Virginia (2.07 μg/g). However, larvae tested from Arkansas ($LC_{50}$ = Farm B, 6.61, Farm C, 10.27 and Farm A, 94.31 μg/g) were less susceptible to permethrin than those tested in Virginia (1.91 μg/g). The Virginia report indicated that permethrin had been applied 3–4 times per week during the house fly season at the commercial egg production poultry house where their test beetles had been collected.

Residual tests - The larval beetles from Farm A had a RR value of 5.2 for cyfluthrin when compared to the residual exposure of the pyrethroid susceptible beetles at Farm B. The larval beetles from Farm C had a RR value of 1.9 when compared to beetles from Farm B indicating that they too were developing resistance to cyfluthrin. The larvae from Farm A were significantly less susceptible (3-times) than larvae from Farm C to permethrin. The adult beetles from Farms A and C were significantly less susceptible to cypermethrin than the adults from the pyrethroid-susceptible population at Farm B and had RR values of 2.8 and 1.5, respectively. The $LC_{50}$ values for the larvae and adults collected from Farm B (used as the susceptible population) were about the same or were slightly more susceptible to cypermethrin than they were to cyfluthrin and permethrin. However adults and larvae from Farm A were ≈2 times more susceptible to cypermethrin than cyfluthrin and permethrin. These data indicated that resistance had developed due to the continuous exposure of the beetle population to pyrethroid-type insecticides over several years.

*Carbamate.* Carbaryl was registered for use in poultry facilities and recommended for the treatment of wood-shaving litter to manage several pests beginning in the 1960s.

Topical application - The beetles from Farm A were resistant to carbaryl (adults >19 times and larvae 2 times) when compared to susceptible beetles from Farm B and the beetles from Farms C and D (Table 1). The adult beetles at Farm

A had a 19 RR for carbaryl compared to the susceptible population from Farm B. The larval beetles from Farms A had 2.6 RR when compared to the susceptible larvae from Farm B. A significant difference existed between the susceptibility of larval beetles from Farm A and D indicating the difference in susceptibility of these populations to carbaryl. The larvae from Farm A, where carbaryl had been used frequently for several years, were significantly less susceptible ($LC_{50}$ = 55.97 µg/g) than the larvae from Farm B ($LC_{50}$ = 31.52) and were also less susceptible to this insecticide than larvae from Farm C ($LC_{50}$ = 21.89 µg/g). The $LC_{50}$ values for adult beetles from the 4 Farms in Arkansas ranged from 0.304 and 0.444 µg/g at Farms C and B, respectively, where no carbaryl had been applied to 8.451 µg/g at Farm A where it had been used extensively. They were more susceptible to carbaryl than adult beetles tested in Virginia ($LC_{50}$ = 128 µg/g, Vaughan & Turner 1984). However, larval $LC_{50}$ values obtained in our tests ranged from 21.89 at Farm C to 55.97 µg/g at Farm A and were close to that reported in Virginia (56.83 µg/g, Vaughan & Turner 1984). Had we not conducted concentration/mortality tests on susceptible beetles from Farm B our $LC_{50}$ values could have been interpreted as representative of carbaryl's effectiveness in causing beetle mortality rather than an indication of resistance caused by carbaryl use. Although data on beetles from only one farm were used in the Virginia study, we noted that the $LC_{50}$ values we obtained for larvae from Farm A, where extensive use of carbaryl had occurred, were almost the same as the Virginia data reported. However, no mention was made of carbaryl use on the farm where the adult and larval beetles were collected during the Virginia study. The $LC_{50}$ values that we obtained from the susceptible population used in the present study indicated that carbaryl did not cause 50% mortality at as low a concentration as the pyrethroid insecticides.

Residual tests - The adult beetles collected at Farm A were significantly more resistant to carbaryl than the beetles from Farms B and C. However, no significant difference was found between the susceptibility of the adults from Farms B and C. As indicated above, carbaryl had been used at Farm A (dust, spray or bait formulation) prior to the start of each flock grow-out over many years.

**Organophosphates** - The application of organophosphate insecticides to the wood-shaving litter of broiler houses to control *A. diaperinus* increased during the mid-1960s and early 1970s. The increase was caused by their involvement as reservoirs of pathogens that cause disease in poultry. Malathion, coumaphos, naled, and dichlorvos were generally recommended for application as sprays.

Topical application - The topical application data showed the impact of previous tetrachlorvinphos use at Farms A and C. The adult and larval beetles were resistant to tetrachlorvinphos at these farms (adult $LC_{50}$ = 0.219 and 0.153, larvae $LC_{50}$ = 9.89 and 10.08 µg/g, respectively) compared to the Farm B susceptible population (adult and larval $LC_{50}$ = 0.059 and 4.01 µg/g, respectively). The larval and adult beetles tested from Farm D were significantly more susceptible to tetrachlorvinphos than the beetles from Farms A and C but were significantly more resistant than the beetles from Farm B. The $LC_{50}$ values for larvae were similar to that of the susceptible population at Farm B and the Virginia larvae (Farm C $LC_{50}$ = 4.01 and Virginia 3.89 µg/g). However, the concentrations required to cause 50% larval mortality were higher at Farms A, C, and D in the Arkansas tests than in the Virginia tests reported in 1984.

Chlorpyrifos was included in these bioassay tests because it had received registeration by EPA for use in poultry facilities and no beetle populations at the farms included in the present study had been exposed to this organophosphate. No significant difference was found between the susceptibility of the adult beetles from Farms B and C and no significant difference was found between the adults from Farms B and D. However, the adults from Farm D were significantly more resistant to chlorpyrifos than the adults from Farm C.

The Farm D larval $LC_{50}$ values for chlorpyrifos were greater than that obtained for larvae treated with tetrachlorvinphos at Farms B and D, but lower than those observed for the larvae from Farms A and C (Table 1).

Residual tests - The impact of prior use of the organophosphates malathion, coumaphos, naled and dichlorvos after the chlorinated hydrocarbons were no longer registered, indicated above, combined with the later use of tetrachlorvinphos and the tetrachlorvinphos-dichlorvos mixture at Farm A are shown in Table 2. The adults and larvae at Farm A were significantly more resistant than the beetle population at Farm C. In addition, the effects of a greater number of years wherein organophosphates were used at Farm A was also apparent. The adult population at Farm A was significantly more resistant to tetrachlorvinphos than the beetles at Farms B and C (9 times). The beetle population at Farm B had not been exposed to insecticides. The bioassay data indicated that they were significantly more susceptible than adult beetles from Farm A and less susceptible than adult beetles from Farm C.

It appears that the tetrachlorvinphos-dichlorvos mixture failures reported in the mid to late 1990s by Arkansas producers could be attributed to the effects of previous dichlorvos and tetrachlorvinphos usage over many years at Farm A and C. These failures in the 3rd, 4th and 5th grow-out each year when beetle numbers were increasing caused even greater concern. Although acceptable beetle control was achieved during the 1st and generally the 2nd flock grow-out, it appeared that similar to the pyrethroids and the carbamate, by the 5th and 6th week of each grow-out the beetle population was increasing significantly. It was not understood why the adult beetles at Farm B were significantly less susceptible than the beetles from Farm C. No significant difference was found in the susceptibility of the larval beetles to chlorpyrifos at Farms A and B, or Farms A and C. The adult and larval beetle populations at Farms A, B and C showed about the same level of susceptibility to chlorpyrifos as they did to tetrachlorvinphos.

The determination of the mechanisms involved in beetle resistance to insecticides along with a greater understanding of the impact of moving beetles throughout the poultry production areas with litter distribution will greatly improve our ability to predict the potential loss of field effectiveness. The relatively close proximity of poultry production facilities containing susceptible and resistant beetles raises interesting questions regarding the rate and amount of population mixing. Savage (1992) reported that litter beetles will fly up to 1.6 km in one night in their movement toward light and he calculated that if litter was distributed into the environment 0.4 km from the house, 6% would fly back in the direction of that house the other 94% disbursing in other directions. It is also important to understand the relationship between adult and larval *A. diaperinus* susceptibility to insecticides within the poultry production areas because only chemicals within the same chemical classes (organophosphate and pyrethroid) are currently being registered for use.

Poultry companies and producers can anticipate the continued loss of field efficacy to the currently registered insecticides if their continued use is not carefully planned. There is a need to develop an integrated management program for the litter beetle that includes new non-chemical strategies. Increased resistance to the few remaining insecticides, environmental concerns, non-renewal of insecticide registration, and a lack of new chemical development significantly threatens the future control of this economically important pest.

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# Comparative Susceptibility of Adult and Larval Lesser Mealworms, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), Collected from Broiler Houses in Arkansas to Selected Insecticides[1]

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**ABSTRACT** Loss of insecticide susceptibility was suspected in lesser mealworms, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), in broiler chicken facilities in Arkansas due to reports of control failures. Bioassay tests using selected insecticides were conducted on adult and larval beetles collected from broiler chicken production farms having different insecticide application history. The residual and topical application tests showed that previous insecticide use had caused significant differences in the relative susceptibility of lesser mealworms. Topical application bioassay tests indicated that adult beetles had greater susceptibility to the insecticides tested than 8th instars collected from the same farm while in residual tests little difference was generally found in the susceptibility of adults and larvae exposed to the insecticides. Data suggested that the ability to de-toxify DDT and other organochlorine insecticides remained in some beetle populations due to spread of wood-shaving litter containing beetles over pastures among broiler production facilities, particularly involving facilities that have been in broiler production for over 40 y. A program utilizing integrated tactics that will reduce the dependency on insecticides is needed to manage litter beetles in poultry production facilities.

**KEY WORDS** litter beetles, lesser mealworm, insecticide susceptibility, *Alphitobius diaperinus*

The number of poultry production facilities being treated with insecticides to manage the lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), continues to increase in response to the poultry industries attempts to prevent significant economic production losses attributed to the presence of the beetles. Initially, the beetle infestations were reported as problematic when the late instars migrated from the wood-shaving or rice hull litter up the facility walls and tunneled into the insulation (Collison 1980) in search of a site in which to molt to the pupal stage followed with the newly emerged adults tunneling out of the insulation. This resulted in extensive economic damage to the insulation (Despins et al. 1987) and resulted in increased heating and cooling costs. Second, starting with the broiler flock initiation with 1-d-old chicks the broilers eat the larval and adult beetles, thus, consuming any

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# Influence of Soybean Maturity Group and Row Width on Bean Leaf Beetle (Coleoptera: Chrysomelidae) and Bean Pod Mottle Disease in an Early Season Production System[1]

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**ABSTRACT** The influence of narrow and wide-row soybeans on infestations of bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae) adults, a vector of bean pod mottle virus (BPMV), and associated incidence of bean pod mottle (BPM) disease were investigated in maturity groups IV and V soybeans in Mississippi. Maturity group IV soybeans had greater cumulative BLB numbers and greater incidence of BPM than maturity group V soybeans in 2000, but not in 2001. Row width was not shown to affect beetle numbers in either study year, but a greater incidence of BPM occurred in narrow row soybeans in 2001 and Maturity Group IV soybeans in 2000. There was no significant correlation between numbers of BLB adults and soybean plants infected with BPM virus when data was analyzed within sample dates or by seasonal totals. Greater yields were obtained in maturity group V soybeans than in maturity group IV soybeans in 2000, but not 2001, whereas row width had no significant effect on yield in either 2000 or 2001. The results presented herein suggest that further investigations of soybean row spacing in relation to BLB and BPM disease should consider large experimental plots to minimize beetle dispersal and spread of BPM disease.

**KEY WORDS** soybeans, planting date, row width, bean leaf beetle, bean pod mottle disease

Row width is one agricultural variable that may be altered by farmers to affect management and economics of soybean production. It has been a common practice in the mid-South in the United States to plant soybeans in wide rows where herbicides may be used effectively to manage weeds (Caviness et al. 1987). Because most farmers produce more than one crop type, soybeans are planted in wide rows so that standard farm equipment may be used for all crops. Due to the advent of more efficient herbicide treatments and Roundup Ready™ varieties, weed management practices are available to allow narrow-row soybean plantings (Mangold 1980). Soybeans planted in narrow rows achieve canopy closure in a shorter period of time than soybeans planted in wide rows, further limiting weed infestation. Narrow-row plantings of soybeans typically yield more than wide-row plantings in years when sufficient moisture is available for plant development

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(Bowers 1995). The primary reason for greater yields in narrow-row soybeans is the earlier time at which canopy closure is attained. Ninety to 95% sunlight interception during late vegetative and early reproductive stages is a prerequisite for maximum soybean yield (Shibles & Weber 1966). Because yield is a function of canopy light interception, and narrow-row soybeans are more efficient in intercepting available sunlight, greater yields may be expected for narrow-row than wide-row plantings (Ablett et al. 1991).

A relatively recent soybean production recommendation includes early planting of an early season maturity group and is referred to as early season production system (ESPS) (Heatherly 1999). The ESPS involves planting soybean maturity groups III or IV in mid-March to mid-April. Planting early and utilizing an early soybean maturity group allows the farmer to harvest much earlier (ca. 1 mo) than if soybeans were planted in mid- to late May. The critical seed-set development stage for soybeans in the ESPS escapes most harmful environmental stresses and relatively high insect pest infestations.

Narrow-row soybean planting practices have an effect on the behavior of some insect pest species (Hamadain & Pitre 2002). The purpose of this study was to determine effects of narrow-row and wide-row planting practices in the ESPS on bean leaf beetle (BLB), *Cerotoma trifurcata* (Förster), adult populations and associated incidence of bean pod mottle (BPM) disease vectored by the beetles.

## Materials and Methods

**2000.** Soybeans were planted on 28 April 2000 using a John Deere MaxEmerge II planter on the Mississippi Agricultural and Forestry Experiment Station Plant Science Research Farm located in Oktibbeha Co., Mississippi. Planting date for this year was later than recommended for ESPS. Roundup Ready™ (RR) varieties included a maturity group IV soybean, Pioneer 9492 RR, and a maturity group V soybean, Pioneer 95B95 RR, each planted in wide and narrow rows. Wide-row plots consisted of 12 rows, 96.5-cm wide and 16.5 m long. Narrow-row plots consisted of 24 rows, 48.3-cm wide and 16.5 m long, to achieve plots of the same total area as the wide-row plots. Four treatments were established with four replications in a randomized complete block design. Roundup Ready varieties were used because they represent current soybean production practices in the mid-South (National Agriculture Statistics Service, 2001). Dual™ (metolachlor, Ceiba-Geigy Corp., Greensboro, NC) herbicide at a rate of 2.8 L/ha, and Roundup (glyphosate, Monsanto, Chesterfield, MO) herbicide at a rate of 2.1 L/ha were applied at planting and at the V6 stage of plant growth (Fehr & Caviness 1977), respectively. Number of plants/m of row was recorded by counting the number of plants in 3.6 m of row.

Sampling for BLB adults from emergence through the V5 stage of plant growth was conducted by visually sampling individual plants in one row on each sample date. Special attention was directed to cotyledonous leaves because this is a preferred feeding location for the beetles. Visual samples were taken weekly from June 7 to 28 and consisted of three subsamples, each 3.6 m of row (10.4 $m^2$ total area) in wide row plots. The sample size in narrow-row plots consisted of two adjacent 3.6 m of row (10.4 $m^2$ total area) and contained approximately twice the number of plants as wide-row plots. A random number generator was used to

determine the row to be sampled, excluding rows sampled in the three previous weeks. The number of BLB adults within each subsample was recorded.

After the V5 growth stage, plants were large enough to be sampled weekly with a sweep net (Kogan et al. 1980). Sweep net sampling, using a 38 cm diam. net, was conducted from July 5 to September 15. Each sample consisted of 36 sweeps (13.2 $m^2$ total area) taken from a randomly selected row in each wide row plot. Two adjacent rows were sampled in narrow-row plots so that the same total area was sampled in wide-row and narrow-row plots. Sampling on adjacent rows was accomplished by drawing the sweep net across both rows. On July 5, sampling was conducted by both visual and sweep net sampling methods to determine if there was a significant difference in efficiency levels of sampling methods. No significant difference in sampling efficacy was observed. Sample areas were marked to prevent resampling within four weeks. Samples were bagged, transported to the laboratory, and the number of BLB adults within each sample was recorded.

Plants within one row (16.5 m) in each wide row plot with apparent symptoms of BPM were visually identified and recorded weekly throughout the growing season. Characteristic mottling of leaves in the upper canopy (top 2–3 nodes) caused by BPMV (Windham & Ross 1985a) was used to visually identify the disease. As with samples for BLB adults, two adjacent rows were sampled for BPM in narrow-row plots in order to obtain the same total sample area. The number of plants with apparent symptoms of BPM was recorded. Sampling was conducted on a weekly basis beginning on June 7, and care was taken to avoid resampling of the same area within individual plots.

To confirm accuracy of identifying BPM diseased plants in the field, soybean leaves were harvested from the upper two nodes of R2 stage (Fehr & Caviness 1977) plants visually identified as positive or negative for symptoms of the disease. The leaf samples were tested for the presence of BPMV using enzyme linked immuno-sorbent assay (ELISA) (AgDia, Elkhart, IN). Ten symptomatic and ten asymptomatic plants were tested. Because symptoms of soybean mosaic disease may appear similar to those of BPM on soybeans, plants were also tested for soybean mosaic virus (SMV) using ELISA procedures.

Yield measurements were taken by harvesting the middle four rows (66.0 row m) of each wide row plot and an equal area within narrow row plots. Plots were harvested using an MXP four-row plot harvester when the seed was at 12–13% moisture. Due to variance in moisture levels, not all treatments were harvested on the same date. The seed was bagged and taken to the laboratory. Each sample was hand cleaned to remove foreign material and seed weight was measured using a standard scale.

Data were analyzed by Proc GLM using SAS v. 8.2 (SAS Institute 2002). Analysis of variance and correlation procedures were conducted on numbers of bean leaf beetles and BPM symptomatic plants. Fisher's protected LSD was used to determine significant differences between numbers of bean leaf beetles, diseased plants, and yield among treatments.

**2001.** Soybean plots in 2001 were located in the same field using the same varieties, treatments, and procedures as in the 2000 study. Wide-row plots consisted of 20 rows, each 96.5 cm wide and 16.5 m long, and narrow-row plots consisting of 40 rows, each 48.3 cm wide and 16.5 m long. All plots were planted on April 10 and Dual and Roundup herbicides were applied as in the 2000 study.

Sampling for BLB adults was initiated on May 4 by visual examination of individual plants in one row on each sample date from emergence through the V5 growth stage. Sample sites within each plot were selected and plant samples were taken as described in the 2000 study. Visual samples consisted of 10.4 $m^2$ of total area within wide row and narrow row plots. Samples were taken to the laboratory and the number of BLB beetle adults was recorded. After the V5 growth stage, samples were taken weekly from June 8 to September 7 using the sweep net sampling method as described in the 2000 study. Sweep net samples consisted of 13.2 $m^2$ of total area within wide-row and narrow-row plots. On June 8, sampling was conducted by both visual and sweep net sampling methods again to determine if significant difference existed between sampling efficiencies. No significant difference in sampling efficacy was observed.

Sampling for BPM diseased plants within plots throughout the growing season was as described in the 2000 study and was initiated on May 25. Accuracy in field identification of BPM and SM was determined using ELISA procedures. Yield measurements were obtained and data were analyzed using SAS v. 8.2 as described in the 2000 study.

## Results and Discussion

There was no significant correlation between numbers of BLB adults and soybean plants infected with BPM virus when data were analyzed within sample dates or by seasonal totals in both 2000 and 2001 (r = 0.0826: 2000 and r = 0.0763: 2001, respectively). Therefore, the data for BLB and BPM diseased soybeans in the two planting systems will be discussed independently.

**2000.** Soybeans averaged 29 plants per $m^2$ in wide row plots and 58 plants per $m^2$ in narrow row plots in 2000.

Adult BLB were first collected on soybeans on June 28, 24 d after emergence of plants in all plots (Fig. 1). At that time, adults in 10.4 $m^2$ of foliage numbered 12 ± 1 in narrow-row, maturity group IV soybeans; 15 ± 1 in wide-row, maturity group IV soybeans; 13 ± 1 in narrow-row, maturity group V soybeans; and 12 ± 1 in wide-row, maturity group V soybeans. Initial beetle infestations were not significantly different among planting systems ($F$ = 1.00; df = 3, 12; $P$ = 0.4262). The low number of BLB collected and their late occurrence may be explained by the behavior of BLB as they emerged from overwintering sites adjacent to the field (Schumm et al. 1983). Soybeans did not emerge at the time of peak BLB emergence from overwintering; therefore, emerging adult beetles dispersed from the study area to soybeans planted earlier in the surrounding areas. Bean leaf beetle peak emergence has been reported to occur in mid-May in Illinois (Jeffords et al. 1983).

Number of BLB adults in narrow-row and wide-row plantings remained similar until August 16, at which time significantly greater numbers were observed in wide-row, maturity group IV soybeans than in other plantings ($F$ = 6.68; df = 3, 12; $P$ = 0.0067). Eleven days later, no significant differences were observed in beetle infestations among planting systems ($F$ = 0.18; df = 3, 12; $P$ = 0.9067). Beetle infestations in narrow row, maturity group IV and the maturity group V soybeans had increased to levels similar to that in wide-row, maturity group IV soybeans.

When the seasonal cumulative numbers of BLB adults were compared, significantly greater numbers of beetles were recorded in maturity group IV than in maturity group V soybeans ($F$ = 4.98; df = 1, 14; $P$ = 0.0425) (Table 1).

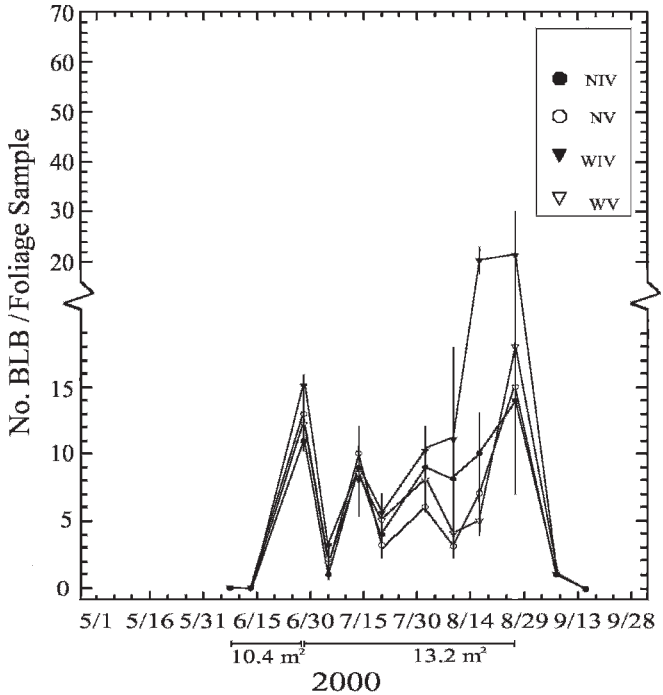


**Fig. 1.** Bean leaf beetle (BLB) population levels in narrow-row maturity group IV (NIV), narrow-row maturity group V (NV), wide-row maturity group IV (WIV), and wide-row maturity group V (WV) soybeans in Oktibbeha Co., Mississippi, 2000.

Interaction between maturity group and planting date was not significant ($F$ = 1.00, df = 1, 12, $P$ = 0.3373). Seasonal cumulative numbers of BLB adults recorded from a study conducted adjacent to this study did not show a significant difference in BLB adults between treatments similar to those described in the present study. The lack of observed differences may have been due to the location of the research plots closer to overwintering sites for BLB, resulting in uniform distribution of beetles throughout the study site. In addition, the greater area of early planted soybeans in the study reported herein may have resulted in the attraction of more BLB adults to the study site.

Symptoms of BPM disease on soybeans in treatment plots were first observed June 28 (Fig. 2). Incidence of disease in maturity group IV soybeans was significantly greater than that in maturity group V soybeans ($F$ = 6.68; df = 3, 12; $P$ = 0.0067). Although greater numbers of diseased plants were observed in maturity group IV plantings than in other planting systems throughout the season, significant differences were not observed after August 10. Further research is needed to describe the relationships among maturity group, row width and incidence of BPM disease. The greater incidence of disease in early-maturing group IV soybeans may reflect greater plant susceptibility in early developmental stages to injury by BPM virus (Windham & Ross 1985a).

**Table 1. Seasonal total number of bean leaf beetle adults, bean pod mottle (BPM) diseased plants and yield for narrow-row, maturity group IV; wide-row, maturity group IV; narrow-row, maturity group V; and wide-row, maturity group V soybeans in Oktibbeha Co., Mississippi, 2000.**

| Main effect | Bean leaf beetles per 13.2 $m^2$ foliage[1] | BPM diseased plants per 16.5 m row[1] | Yield (kg/ha) |
|---|---|---|---|
| Row width | | | |
| Narrow | 71 ± 10 a[2] | 155 ± 17 a | 396 ± 47 a |
| Wide | 84 ± 10 a | 164 ± 17 a | 444 ± 47 a |
| Maturity group | | | |
| IV | 86 ± 10 a | 188 ± 17 a | 324 ± 47 b |
| V | 69 ± 10 b | 132 ± 17 b | 516 ± 47 a |

[1]Means in a column within main effect followed by the same letter are not significantly different ($P < 0.05$).
[2]Least square means (±SE).

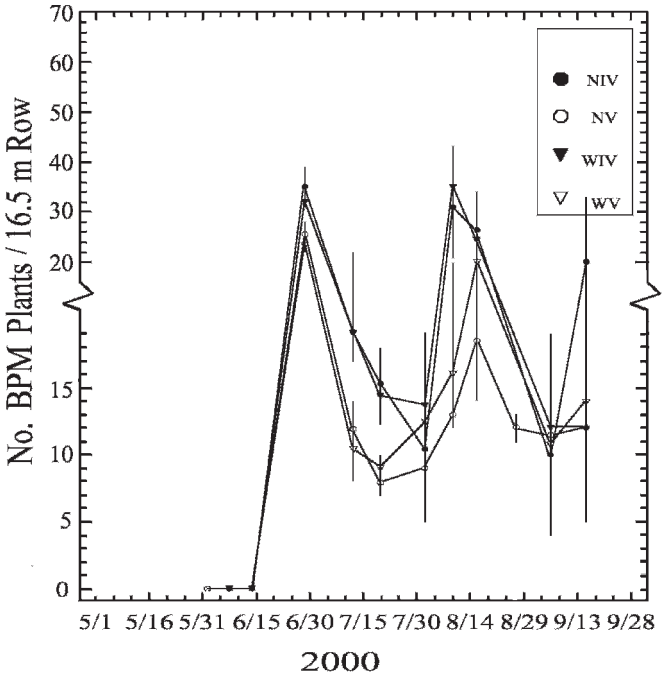


**Fig. 2.** Incidence of bean pod mottle (BPM) diseased plants in narrow-row maturity group IV (NIV), narrow-row maturity group V (NV), wide-row maturity group IV (WIV), and wide-row maturity group V (WV) soybeans in Oktibbeha Co., Mississippi, 2000.

When seasonal cumulative total incidence of BPM diseased soybean plants within plots was analyzed, neither maturity group nor planting date main effects were significant (Table 1). Interaction between maturity group and planting date was also not significant ($F = 0.0$; df = 1, 12; $P = 1.00$).

Testing by ELISA for accuracy in disease identification in the field showed a 90% level of accuracy in selecting BPM diseased plants and a 100% level of accuracy in selecting non-infected plants; no plants were determined to be infected by SMV.

There was no significant effect of row-width on yield ($F = 6.0$; df = 1, 14; $P = 0.4519$) (Table 1). However, significantly greater yield was obtained from maturity group V than maturity group IV soybeans ($F = 48.39$; df = 1, 14; $P < 0.0001$). Interaction between maturity group and planting date was not significant ($F = 0.17$; df = 1, 12; $P = 0.6894$). The greater yields for maturity group V soybeans may be due to the relatively late planting in 2000. Dry conditions during the critical growth stages of maturity group IV soybeans did not provide optimum conditions for plant development. According to rainfall measurements recorded at the Mississippi Agriculture and Forestry Experiment Station (MAFES), the total growing season (May–September) rainfall for 2000 was 21.5 cm; this was 24.1 cm below the 10 y seasonal average of 45.6 cm for the study site (MAFES, unpublished). Rainfall was particularly low during the late vegetative and early reproductive growth stages of soybeans in August and September in this study.

**2001.** Soybeans averaged 29 plants per $m^2$ in wide row plots and 58 plants per $m^2$ in narrow row plots in 2001.

In 2001, BLB adults were first observed on May 4, 25 d after plant emergence (Fig. 3). Greater numbers of beetles on soybeans in 2001 (Table 2) than in 2000 (Table 1) can be attributed to greater numbers of overwintering beetles colonizing the earlier planted soybeans in 2001 as compared with the later planting in 2000. Number of BLB adults in maturity group IV soybeans on the initial sampling date was significantly greater than in maturity group V soybean plots ($F = 3.47$; df = 3, 12; $P = 0.0509$) (Fig. 3). This difference may reflect an initial BLB preference for maturity group IV soybeans over maturity group V soybeans. However, this difference did not persist after the first sampling date. As in the 2000 study, in the same field, the initial population levels in treatment plots declined after the first week of sampling. This decline may have been due to natural mortality of the overwintering population of adults.

Significantly greater numbers of BLB adults were recorded in wide row, maturity group IV and V soybeans than in narrow row, maturity group IV soybeans on July 11 ($F = 2.19$; df = 3, 12; $P = 0.0036$) after which the beetle population declined sharply. This decline in beetle numbers may be related to natural adult mortality in the second generation. The population levels of BLB adults remained low after this time.

The cumulative seasonal total of BLB adults revealed that there was no significant difference in numbers of beetles in narrow row planted soybeans and wide row planted soybeans (Table 2). Also, maturity group did not significantly affect numbers of adult BLB in 2001 ($F = 0.39$; df = 1, 12; $P = 0.5431$). Interaction between maturity group and planting date was not significant ($F = 0.03$; df = 1, 12; $P = 0.8686$). Seasonal cumulative numbers of BLB adults recorded from a study conducted adjacent to this study showed significantly greater numbers of BLB adults in Maturity Group IV soybeans than in Maturity

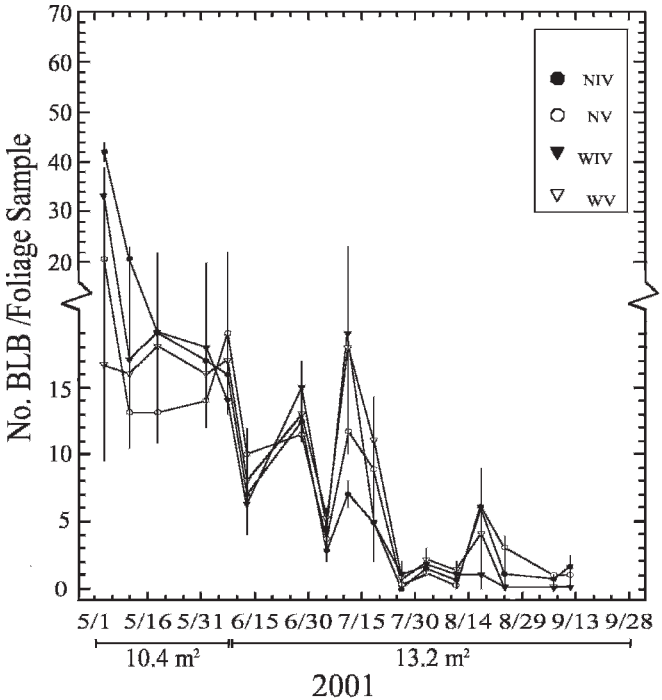


**Fig. 3.** Bean leaf beetle (BLB) population levels in narrow-row maturity group IV (NIV), narrow-row maturity group V (NV), wide-row maturity group IV (WIV), and wide-row maturity group V (WV) soybeans in Oktibbeha Co., Mississippi, 2001.

**Table 2. Seasonal total number of bean leaf beetle adults, bean pod mottle (BPM) diseases plants, and yield for narrow-row, maturity group IV; wide-row, maturity group IV; narrow-row, maturity group V; and wide-row maturity group V soybeans in Oktibbeha Co., Mississippi, 2001.**

| Main effect | Bean leaf beetles per 13.2 $m^2$ foliage[1] | BPM diseased plants per 16.5 m row[1] | Yield (kg/ha) |
|---|---|---|---|
| Row width | | | |
| Narrow | 116 ± 10 a[2] | 178 ± 22 a | 557 ± 53 a |
| Wide | 132 ± 10 a | 82 ± 22 b | 568 ± 53 a |
| Maturity group | | | |
| IV | 126 ± 10 a | 136 ± 22 a | 581 ± 53 a |
| V | 123 ± 10 a | 124 ± 22 a | 544 ± 53 a |

[1]Means in a column within main effect followed by the same letter are not significantly different ($P < 0.05$).
[2]Least square means (±SE).

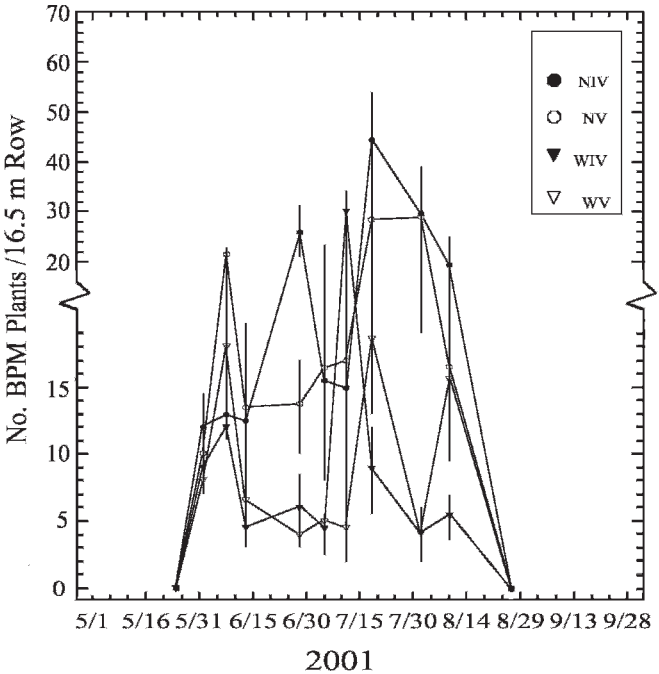


**Fig. 4.** Incidence of bean pod mottle (BPM) diseased plants in narrow-row maturity group IV (NIV), narrow-row maturity group V (NV), wide-row maturity group IV (WIV), and wide-row maturity group V (WV) soybeans in Oktibbeha Co., Mississippi 2001.

Group V soybeans. The greater number of beetles in Maturity Group V soybeans in contrast to Maturity Group IV soybeans in the adjacent study may have been due to the plants being more attractive to the beetles as they emerge from overwintering. Greater attractiveness of Maturity Group V soybeans may have been due to continuing vegetative growth. This preference may have been weaker as beetles dispersed throughout the study area.

Symptoms of BPM disease on young soybeans were first observed June 1 (Fig. 4). No significant differences in incidence of diseased plants were recorded at this time. In the following week, a significantly greater number of diseased plants was observed in narrow-row, maturity group V soybeans than in narrow-row, maturity group IV or wide-row maturity group IV soybeans ($F$ = 2.92; df = 3, 12; $P$ = 0.0776).

A greater number of BPM diseased plants were observed in narrow-row soybean plantings of maturity groups IV and V on June 28, July 5, and August 2 ($F$ = 6.68; df = 3, 12; $P$ = 0.0067: $F$ = 1.54; df = 3, 12; $P$ = 0.0123; $F$ = 7.32; df = 3, 12; $P$ = 0.0048, respectively)than in wide-row plantings (Fig. 4). The greater incidence of BPM disease in narrow-row soybeans may be accounted for by the higher concentration of plants within these plots compared with wide-row plots of equal planted area. The close association of these plants could allow for greater movement of viruliferous beetles from plant to plant and row to row.

Observations of BPM disease incidence throughout the sample period were characterized by high variance in observed numbers of diseased plants within sampling dates and fluctuations in observed numbers of diseased plants from sampling date to sampling date. This variance in disease data was apparently due to a clumped distribution of diseased plants in the study area. This clumped distribution occurred as beetles acquired the virus from feeding on infected soybean plants and then moving to adjacent plants to feed, spreading the virus (Windham & Ross 1985a).

The seasonal cumulative total incidence of BPM diseased plants within plots revealed that the number of diseased plants in narrow-row soybeans was significantly greater than the number of diseased plants observed in wide-row soybeans ($F$ = 16.9; df = 3, 12; $P$ = 0.0001) (Table 2). Interaction between maturity group and planting date was not significant ($F$ = 0.13, df = 1, 12, $P$ = 0.7294).

Testing by ELISA for accuracy in disease identification in the field as in the 2000 study indicated a 100% level of accuracy in identifying BPM infected plants and a 90% level of accuracy in identifying non-infected plants; no plants were determined to be infected by SMV.

Neither soybean maturity group nor row width main effects influenced yield in the 2001 study (Table 2). Interaction between maturity group and planting date was not significant ($F$ = 3.35; df = 1, 12; $P$ = 0.092). The greater yields in 2001 compared with 2000 may be attributed to the greater amount of rainfall at the study site in 2001 (155 cm, which is 28 cm greater than the ten year average of 127 cm and 55 cm greater than in the previous season). In 2000, lowest levels of rainfall occurred during July and August when maturity group IV soybeans were entering the reproductive stages of plant development. Row width was not shown to significantly affect soybean yield in this study. It is reasonable to believe that plant growth stage, condition of plants when infested with BLB adults, and plant infection with BPM are factors that could influence soybean yield (Windham & Ross 1985b, Hunt et al. 1995). A limitation in this row spacing study appeared to be the size of soybean treatment plots, because of the ability of bean leaf beetles to disperse within crop production areas. Larger experimental areas would minimize vector beetle movement and spread of BPM virus from plot to plot. This would be particularly significant during the vegetative growth stages of soybean varieties of different maturity groups, when plants are most susceptible to infection by BPM virus. Small plots do not limit to any significant degree the movement of vector beetles from plot to plot throughout the developmental stages of soybean plants of different maturity groups. Dispersal would be most apparent when early maturing soybeans become less attractive than later maturing soybeans growing nearby and in more attractive vegetative stages. Further investigations of soybean row spacing in relation to BLB and BPM disease should consider larger experimental plots than those included in this study.

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# Influence of Soybean Maturity Group and Row Width on Bean Leaf Beetle (Coleoptera: Chrysomelidae) and Bean Pod Mottle Disease in an Early Season Production System[1]

Ronald C. Stephenson and Henry N. Pitre[2]

J. Agric. Urban Entomol. 25(2): 127–137 (April 2008)

**ABSTRACT** The influence of narrow and wide-row soybeans on infestations of bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae) adults, a vector of bean pod mottle virus (BPMV), and associated incidence of bean pod mottle (BPM) disease were investigated in maturity groups IV and V soybeans in Mississippi. Maturity group IV soybeans had greater cumulative BLB numbers and greater incidence of BPM than maturity group V soybeans in 2000, but not in 2001. Row width was not shown to affect beetle numbers in either study year, but a greater incidence of BPM occurred in narrow row soybeans in 2001 and Maturity Group IV soybeans in 2000. There was no significant correlation between numbers of BLB adults and soybean plants infected with BPM virus when data was analyzed within sample dates or by seasonal totals. Greater yields were obtained in maturity group V soybeans than in maturity group IV soybeans in 2000, but not 2001, whereas row width had no significant effect on yield in either 2000 or 2001. The results presented herein suggest that further investigations of soybean row spacing in relation to BLB and BPM disease should consider large experimental plots to minimize beetle dispersal and spread of BPM disease.

**KEY WORDS** soybeans, planting date, row width, bean leaf beetle, bean pod mottle disease

Row width is one agricultural variable that may be altered by farmers to affect management and economics of soybean production. It has been a common practice in the mid-South in the United States to plant soybeans in wide rows where herbicides may be used effectively to manage weeds (Caviness et al. 1987). Because most farmers produce more than one crop type, soybeans are planted in wide rows so that standard farm equipment may be used for all crops. Due to the advent of more efficient herbicide treatments and Roundup Ready™ varieties, weed management practices are available to allow narrow-row soybean plantings (Mangold 1980). Soybeans planted in narrow rows achieve canopy closure in a shorter period of time than soybeans planted in wide rows, further limiting weed infestation. Narrow-row plantings of soybeans typically yield more than wide-row plantings in years when sufficient moisture is available for plant development

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# Sampling Methods in Evaluating Crape Myrtle Aphid *Tinocallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae) Densities Versus Time[1]

Vassilis A. Vassiliou[2] and Bastiaan "Bart" M. Drees[3]

J. Agric. Urban Entomol. 25(2): 139–144 (April 2008)

**ABSTRACT** A comparison is made of the relative efficiency of three sampling methods for crape myrtle aphid: the number of aphids of 12.7 cm terminal sample, the number of aphids on five randomly selected leaves on 12.7 cm terminal sample, and aphids on three most infested leaves per terminal sample.

The crape myrtle aphid (CMA), is one of the key insect pests of crape myrtle, *Lagerstroemia indica* L., and was apparently introduced into the United States with crape myrtle, its host plant (Mizell & Schiffhauer 1987). CMA is pale yellow in color with winged adults having black wings and black protuberances. They primarily are found on undersides of leaves and are particularly attracted to new growth. CMA is not found on any other commonly grown plant. No aphid species other than CMA aphid infest crape myrtle in Texas (Drees & Jackman 1998).

This species damages crape myrtle by inserting mouthparts into soft tissue and extracting plant sap. Heavy infestations distort leaves and stunt new growth. During feeding, aphids secrete droplets of a sugary solution called "honeydew." Drops of honeydew fall from the aphids onto leaves and stems below. This sugary solution promotes the growth of sooty mold fungi, *Capnodium* sp. Sooty mold appears as a black staining or powdery coating on leaves and stems. The blackened leaves and stems are often the most obvious sign of aphid infestation. The black fungus shades the leaves and interferes with photosynthesis, potentially reducing the long-term vigor of the plant.

CMA can reproduce and develop large numbers rapidly. In Texas, populations generally peak between late June and early August (Drees & Jackman 1998). In North Carolina aphid populations remain through October (Baker 1980).

Crape myrtles should be inspected regularly during this period to monitor populations of aphids. Although many predatory insects feed on crape myrtle aphids, they usually cannot control the aphids. Aphid populations can probably be managed if control measures begin by the first week of July. Foliar sprays of

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insecticidal soaps or horticultural oils are the most environmentally safe pesticides for controlling crape myrtle aphids (Drees & Jackman 1998). Control of CMA will halt further development of sooty mold.

The distribution of aphids on most plants is aggregated typical of many insects. In general, aphids prefer to feed and reproduce on young growing parts compared to mature parts. Further, the aphids that aggregate set different problems from those that disperse. Aggregates of aphids are usually easier to find than widely spaced species, but it is more difficult to estimate their numbers.

Counting the number of aphids per plant is a time-consuming process and requires specialized personnel (Ward et al. 1985). In order to monitor aphid populations, a time-efficient sampling plan is necessary. Correct identification and efficient sampling methods for various insect pests are two critical steps toward implementation of Integrated Pest Management (IPM) programs. Efficient sampling methods are necessary for making accurate and timely correct evaluations of insect population in an area or infestation levels. These estimates can then be used for comparison with economic thresholds and consideration of appropriate management tactics (Fettig et al. 2005).

Sampling is one of the most important procedures that are used in any IPM program. Many researchers have devoted significant effort to the theory, development, and practical implementation of sampling methods (Fettig et al. 2005). The development of sampling programs for insect pests is an extensive area of research (Southwood 1978, Pedigo & Buntin 1994). According to Pedigo (1989), this area of research consists of two basic components: the sampling technique and sampling program. A sampling program describes the procedures that to be followed for deploying the sampling technique. A typical sampling program describes the sample unit, the most appropriate insect stage to sample, the number of samples to be collected, the appropriate timing of conducting the sampling, and the pattern of sampling, i.e., at random or systemic (Southwood 1978, Pedigo 1989, Pedigo & Buntin 1994, Fox et al. 1997).

The main goal of this study was to compare the efficacy of various CMA sampling methods in landscape grown crape myrtles and the time required for such sampling techniques. These sampling methods will be helpful in evaluating the efficacy of applied insecticides in post-treatment counts compared to aphid pre-treatment densities. Results of conducting multiple sampling methods for species characterized by high population densities are intended to help applied researchers better understand such methods. In addition, the most convenient methods will be available to design, sample, conduct and evaluate insecticide effectiveness or efficacy studies, and, finally, save valuable time that is usually spent ineffectively on pre-treatment counts.

## Materials and Methods

**Location and material.** All sampling methods were carried out at the Riverside Campus of Texas A&M University, College Station, Texas, on aphid-infested crape myrtle shrubs of unknown variety. This study was conducted 10 August 2007, and all sampling methods were demonstrated on three crape myrtle shrubs of about the same size. For each sampling method, the time required for obtaining aphid counts was recorded.

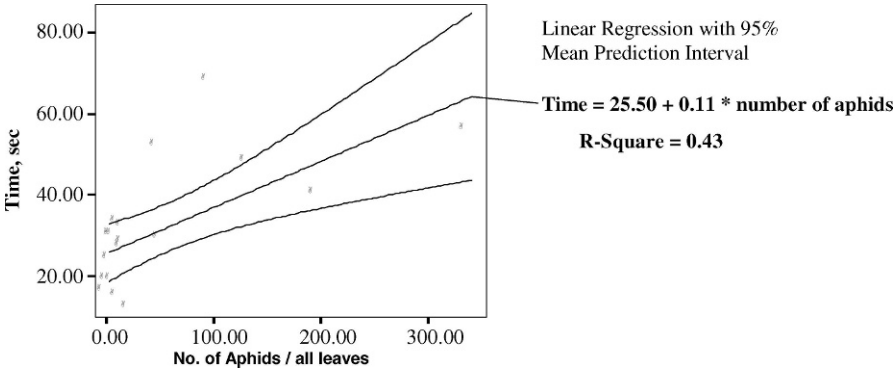


**Fig. 1.** Number of aphids on leaves of 12.7 cm terminal sample and time required for such counts ($R^2$ Adjusted = 0.392; $F_{1,16}$ = 11.943; $P \leq 0.003$; Mean sq. = 1708.656).

## Sampling methods

**Method 1. Number of aphids on leaves of 12.7 cm terminal sample.** Six randomly selected 12.7 cm terminals, containing an average of 26 leaves, were examined without cutting them for crape myrtle aphid presence. The approximate number of aphid nymphs on both sides of each leaf within the terminal sample length was recorded.

**Method 2. Number of aphids on five randomly selected leaves on 12.7 cm terminal sample.** For this method, the number of aphids on both sides of five randomly selected leaves of a 12.7 cm terminal sample was recorded. The 12.7 cm terminals were examined without cutting them.

**Method 3. Aphids on three most infested leaves per terminal sample.** This method of pre-treatment counts was proposed by J.A. Reinert (1976). Aphid counts were performed on three most infested leaves on each of the six randomly selected 12.7 cm terminal samples without cutting them. The time required for the detection of these sample leaves on the sample terminal was calculated after the three more aphid-infested leaves were found.

**Data analysis.** Aphid and time means for each sampling method were subjected to an analysis of variance (ANOVA), with the means separated using Tukey's Honestly Significant Difference, at $P \leq 0.05$. Linear regression analysis between aphid number (independent) and time (dependent) required for was performed at $P \leq 0.05$ (SPSS for Windows, Version 14.0).

## Results

*Sampling method 1.* There was a linear relationship ($r^2$ = 0.43) between aphid numbers, and time required for these counts using the aphid-infested leaves of 12.7 cm terminal sample ($R^2$ Adjusted = 0.392; $F_{1,16}$ = 11.943; $P \leq$ 0.003; Mean sq. = 1708.656), where Time = 25.50 + 0.11 × number of aphids (Fig. 1).

*Sampling method 2.* Linear relationship was also observed ($r^2$ = 0.883) between aphids counted on 5 leaves, and time required for such counts using the

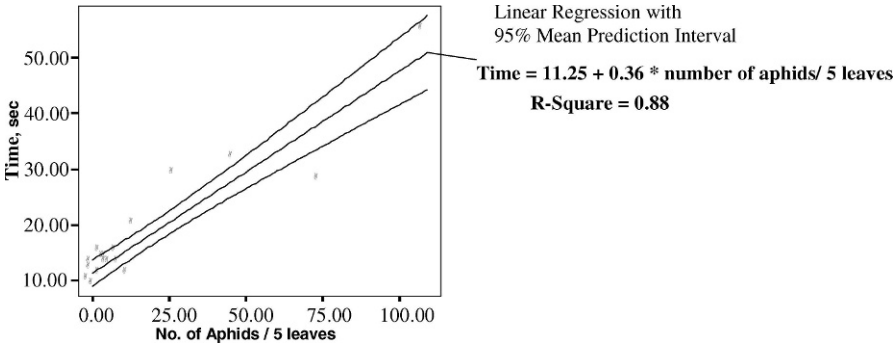


**Fig. 2.** Number of aphids on five randomly selected leaves of 12.7 cm terminal sample and time required for such counts ($R^2$ Adjusted = 0.875; $F_{1,16}$ = 120.307; $P \leq 0.000$; Mean sq. = 1958.088).

5 randomly selected leaves of 12.7 cm terminal sample ($R^2$ Adjusted = 0.875; $F_{1,16}$ = 120.307; $P \leq 0.000$; Mean sq. = 1958.088), where Time = 11.25 + 0.36 × number of aphids/5 leaves (Fig. 2).

*Sampling method 3.* Analysis of regression revealed a strong linear relationship ($r^2$ = 0.922) between the numbers of aphids counted on three most infested leaves of a 12.7 cm terminal sample, and time required for such counts ($R^2$ Adjusted = 0.918; $F_{1,16}$ = 190.189; $P \leq 0.000$; Mean sq. = 3080.189), where Time = 8.80 + 0.37 × number of aphids/ 3 leaves (Fig. 3).

## Discussion

A very common problem for most farmers, urban landscapers and plant protection specialists is in deciding whether or not to apply insecticides in order to control a specific insect pest in a certain crop or ornamental plants. Integrated Pest Management programs aim to reduce losses associated with insect pests to

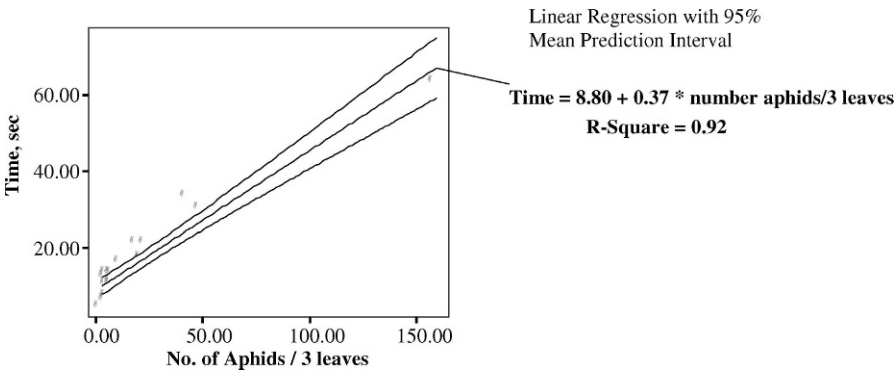


**Fig. 3.** Number of aphids on three most infested leaves of 12.7 cm terminal sample and time required for such counts ($R^2$ Adjusted = 0.918; $F_{1,16}$ = 190.189; $P \leq 0.000$; Mean sq. = 3080.189).

acceptable levels. In order to achieve this, several economically viable, effective, ecologically compatible and time saving techniques and methods are used (Fettig et al. 2005).

Decision making is a key aspect of any Integrated Pest Management Program and will continue to play a significant role as IPM programs mature. Sampling is a very important tool for the assessment of insect pest density which usually requires obtaining actual counts of the pest (Binns & Nyrop 1992).

*Sampling methods.* A sampling technique is a method used to collect information from a single sampling unit. The focus of a sampling technique is on equipment and/or the way an arthropod count is accomplished. A sampling program comprises two types, extensive programs and intensive programs (Pedigo & Buntin 1994).

Population sampling estimates the current insect population in the stand and the level of damage that can be expected. Population sampling techniques determine the current population of insects based on presence of life stages in samples. Actively feeding early and late instars are sampled to determine the current insect population. This is very important to verify the necessity of applying direct control measures and to evaluate their efficacy.

Sampling techniques verify population levels and confirm treatment areas in the spring to early summer against various insect pests. Population sampling is also done during the major feeding period to assess efficacy of control programs.

Aphids have high reproductive potential leading to extremely high population densities. Their population densities and distribution can be documented using various sampling techniques that can be useful evaluating the efficacy of various insecticides over time. CMA population density and distribution on their host crape myrtles vary over time by variety and location. Sampling all leaves on 12.7 cm terminals proved to be a helpful tool in assessing the aphid population density (Method 1). This method provided more precise evaluation of the population density on the sampled leaves, but it required much more time.

The aphid-infested leaves per 5-leaf sample technique (Method 2) required less time compared to Method 1 in recording aphid numbers on sample leaves, but failed to show more subtle differences due to less variability of numerical estimates of aphid numbers generated. Finally, sampling the three most infested leaves proved to be time-efficient and resulted in the highest $R^2$ value for the linear regression (Figs. 1–3). In these cases, even one aphid nymph was presented on the treated sampled leaves was considered as infested, minimizing the estimation on the actual aphid density of the sample. Conversely, in instances where low population densities occur in applied research, sampling more plant material more quickly may overcome low counts or zero data points in the evaluation. Results obtained from the three sampling techniques used here can contribute to blocking from the highest to the lowest aphid numbers and assigning treatments to replicates, providing more uniformity between treatment means.

*Statistical method used.* Statistical procedures are a combination of logic and arithmetic that allow us to interpret information gathered from experiments. The purpose of these experiments was to evaluate the effectiveness of various insecticides and insecticide rates on crape myrtle aphid, and compare the mean of each of these experimental groups with the mean of a control group. In this case, an Analysis of Variance (ANOVA) was used in order to separate the means. In

our trials, we have employed Tukey's Honest Significant Difference to separate the means.

Tukey's Honest Significant Difference is used for testing the significance of unplanned pairwise comparisons. When we use multiple significant tests, the chance of finding a significant difference just by chance increases. Tukey's Test is one of the methods of ensuring that the chance of finding a significant difference in any comparison under a null model is maintained at alpha level of the test.

Linear Regression was essential to analyze the relationship between aphid numbers versus time. The goal here is to adjust the values of slope and intercept to find the line that best predicts Y from X, and minimize the sum of squares of the vertical distances of the points from the line. The higher the aphid numbers the more the time required. Because the linear regression equation generated is significant (Fig. 1 and 2), any point along the line is significantly different from any other point along the line.

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# Sampling Methods in Evaluating Crape Myrtle Aphid *Tinocallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae) Densities Versus Time[1]

Vassilis A. Vassiliou[2] and Bastiaan "Bart" M. Drees[3]

J. Agric. Urban Entomol. 25(2): 139–144 (April 2008)

**ABSTRACT** A comparison is made of the relative efficiency of three sampling methods for crape myrtle aphid: the number of aphids of 12.7 cm terminal sample, the number of aphids on five randomly selected leaves on 12.7 cm terminal sample, and aphids on three most infested leaves per terminal sample.

The crape myrtle aphid (CMA), is one of the key insect pests of crape myrtle, *Lagerstroemia indica* L., and was apparently introduced into the United States with crape myrtle, its host plant (Mizell & Schiffhauer 1987). CMA is pale yellow in color with winged adults having black wings and black protuberances. They primarily are found on undersides of leaves and are particularly attracted to new growth. CMA is not found on any other commonly grown plant. No aphid species other than CMA aphid infest crape myrtle in Texas (Drees & Jackman 1998).

This species damages crape myrtle by inserting mouthparts into soft tissue and extracting plant sap. Heavy infestations distort leaves and stunt new growth. During feeding, aphids secrete droplets of a sugary solution called "honeydew." Drops of honeydew fall from the aphids onto leaves and stems below. This sugary solution promotes the growth of sooty mold fungi, *Capnodium* sp. Sooty mold appears as a black staining or powdery coating on leaves and stems. The blackened leaves and stems are often the most obvious sign of aphid infestation. The black fungus shades the leaves and interferes with photosynthesis, potentially reducing the long-term vigor of the plant.

CMA can reproduce and develop large numbers rapidly. In Texas, populations generally peak between late June and early August (Drees & Jackman 1998). In North Carolina aphid populations remain through October (Baker 1980).

Crape myrtles should be inspected regularly during this period to monitor populations of aphids. Although many predatory insects feed on crape myrtle aphids, they usually cannot control the aphids. Aphid populations can probably be managed if control measures begin by the first week of July. Foliar sprays of

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# Bean Leaf Beetle (Coleoptera: Chrysomelidae) Response to Soybean Variety and Organic-Compliant Treatments in Iowa[1]

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J. Agric. Urban Entomol. 25(3): 145–163 (July 2008)

**ABSTRACT** In response to concerns from certified organic producers who were experiencing significant market losses due to seed staining of soybean [*Glycine max* (L.) Merr.], we evaluated alternative ways to manage bean leaf beetles [*Cerotoma trifurcata* (Forster)] (Coleoptera: Chrysomelidae), a known vector for the seed-staining bean pod mottle virus (BPMV). From 2000 through 2006, organic-compliant treatments, including insecticidal and soil fertility products in use by organic farmers, were compared in on-farm and experiment-station trials. Two soybean varieties, Northrup-King 2412 (NK2412) and Pioneer Brand 9305 (P9305), also were evaluated for bean leaf beetle populations. Overall, the NK2412 variety hosted fewer beetles although there was not a significant yield effect. None of the organic-compliant treatments provided measurable control of bean leaf beetle populations, nor did they affect beneficial insect populations. Organic soybean yields ranged from 1.8 to 3.7 Mg $ha^{-1}$ across all years with no effect from treatments. Producers are encouraged to select soybean varieties based on insect pest response and to monitor bean leaf beetle populations to determine the effectiveness of this strategy in organic systems.

**KEY WORDS** bean leaf beetle, *Cerotoma trifurcata* Coleoptera, Chrysomelidae, soybean, *Glycine max* (L.) Merrill, seed yield, seed composition, seed staining

During the 1999 growing season, certified organic producers in Iowa requested research assistance when stained soybean [*Glycine max* (L.) Merr.] seeds were rejected from food-grade markets (e.g., tofu) at a rapidly increasing rate. Seed coat staining resulted from infection by bean leaf beetle-vectored bean pod mottle virus (BPMV), purple stain [*Cercospora kikuchii* (Mastsumoto & Tomoyasu) M.W. Gardener] and *Fusarium* spp. (Stace-Smith 1981, Lin & Hill 1983, Krell et al. 2004).

Bean leaf beetles generally have two generations in Iowa, with over-wintering adults from the previous year's second generation feeding primarily on vegetative stages of soybean (Rice & Pope 2007). Using a threshold of 7.8°C, first-generation

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adults require 1212 degree-days to develop, and usually achieve peak populations when soybean is in the early reproductive growth stages (Lam et al. 2001). Second-generation adults, whose numbers are dependent on the first-generation population size, achieve peak populations during the pod-filling stage. Feeding by first-generation beetles on soybean leaves seldom results in economic yield losses, but when the second-generation adults emerge from the soil to feed on seedpods, crop damage in late summer can be significant (Lam et al. 2001). The second-generation adults overwinter in the soil and leaf litter where they remain until spring of the following year. The severity of the over-wintering period is a key factor in determining insect survival, with snow cover (Lam & Pedigo 2000a) and woodland areas (Lam & Pedigo 2000b) aiding survival.

Both bean leaf beetle generations can transmit the BPMV, although disease incidence is generally greater during pod setting and filling because higher amounts of rainfall often create ideal conditions for spreading the disease (Krell et al. 2003). Infected plants can be characterized by yellow to green blotchy leaves that can have a raised or blistered appearance and seed having seed staining or hilum bleeding (Hill et al. 2006). BPMV has been reported to cause yield losses >50% (Hopkins & Mueller 1984) and, in 1999, it was estimated that soybean yield losses reached 155,778 metric tons in Iowa due to soybean viruses (Wrather et al. 2003). Management of bean leaf beetles is recommended as soon as soybean cotyledons emerge, as there is a strong positive relationship between plant age at infection and yield reduction (Ragsdale 1984).

The predominant soybean varieties for tofu and soymilk markets were bred for a large seed size and specific protein content. The Japanese market, a mainstay for organic soybean producers, also prefers a white seed color (L. A. Wilson, Iowa State Univ., personal communication). Reducing the extent of soybean staining is of great economic importance to organic producers because of the premium prices associated with unstained seed. Furthermore, in addition to seed quality impacts, infestations of bean leaf beetles impact soybean yields, as Hunt et al. (1994) determined that leaf defoliation of 68% in seedling soybeans caused 12% reduction in yield. Bean leaf beetle feeding also can cause up to 40% soybean pod damage (Witkowski & Echtenkamp 1996).

For organic soybean producers, cultural pest management strategies for bean leaf beetle included tillage, delayed planting and soil organic matter management, despite limited research on these tactics. Phelan et al. (1995) reported that egg-laying by *Ostrinia nubilalis* (Hubner) was reduced on corn grown in organic–amended soils compared to soils from conventional farms, but there were no differences detected in beneficial insect populations. In other organic studies, leaf- and plant-hoppers were reduced by high fertility treatments (Luna 1988, Kajimura et al. 1993). Results with flea beetles (*Phyllotreta* spp.) were mixed: beetles were greatest on collards (*Brassica oleracea*) amended with sewage sludge early in the season but then the reverse occurred at the end of the season (Culliney & Pimentel 1986). Tillage was found to be effective in mitigating stink bug (*Nezara viridula* L.) damage and increasing predatory populations of *Geocoris* spp. on soybean compared to no-tilled plots (Buntin et al. 1995). Delayed planting has also been recommended to mitigate bean leaf beetle, as Zeiss & Pedigo (1996) determined that a longer visitation on alternate crops led to reduced lifespan and reproductive capacity on soybean. Beetles on alfalfa, however, were able to compensate for delayed soybean feeding. Planting in mid-

May is recommended for reducing initial virus transmission (Bradshaw & Rice 2003).

The organic farmers who brought the seed staining problem to the attention of USDA-ARS and university scientists at the annual Practical Farmers of Iowa meeting in February 2000 reported they were using several organic-compliant pest management treatments to help manage bean leaf beetles and reduce transmission of virus or fungal agents responsible for seed coat staining. There was virtually no information to help guide their decision-making, a problem exasperated by regulations governing organic production that require the use of an integrated systems approach to pest management (USDA-AMS 2008a). Increased tillage is discouraged because enhancement of soil organic matter is required to meet certified organic standards. Furthermore, the rules emphasize reliance on biological insect control to manage most insect pests. Thus, natural enemies of the bean leaf beetle should not be negatively affected by any materials applied for insect pest control. Rather, a healthy soil that supports beneficial soil microbial populations for improved plant health and increased pest resistance or tolerance (Merrill 1983) should be maintained. Natural enemies of bean leaf beetle include mites [*Trombidium hyperi* (Acari: Trombidiidae)] (Peterson et al. 1992) on bean leaf beetle larvae and the parasitic fly, *Medina* sp. nov. (Diptera: Tachinidae) (Loughran & Ragsdale 1986).

The objective of these experiments was to quantify effects of organic–compliant pest management treatments in use by organic farmers to manage bean leaf beetles, soybean staining, and improve crop yield. Both insecticidal and soil fertility products were evaluated. Specific products tested over this 7-y study varied but were always based on recommendations by the Organic Agriculture Advisory Committee who met annually to review results and recommend changes, including the use of new products reported by growers to have efficacy against bean leaf beetles. Two soybean varieties commonly grown by organic producers in Iowa were evaluated to determine if there were differences in bean leaf beetle feeding and the subsequent degree of seed staining.

## Materials and Methods

**On-farm soybean variety trial.** Two soybean varieties, Pioneer Brand 9305 (P9305) and Northrup-King 2412 (NK2412), were planted on certified organic farms near Fontanelle, Harlan, Norway, Pella, and Peosta, Iowa, in 2000. A row-spacing of 76 cm and seeding rate of 494,000 seed $ha^{-1}$ were used at all locations. The two varieties were planted in a completely randomized design of 8 four-row (6.08 m) by 61-m long strips, typical of on-farm research design (Exner & Thompson 2003). Planting dates for the five sites were 23, 21, 19, 22, and 22 May, respectively. Bean leaf beetle and other insect populations were measured several times during the growing season (Table 1). In-situ counts of bean leaf beetles were made when soybeans were in the VE–V4 stages by examining every plant along 3 m of row within the 3 middle rows of each strip. All insect sampling was done inside a buffer of at least 5 m from any field edge to ensure an accurate measure of insect populations. At the V4 soybean growth stage, sweep-net samples (20 sweeps per sample) were taken within a single row in the middle of each replicated strip plot using a 38-cm diameter sweep net. A different row was sampled each period. Samples were placed in Ziplock™ bags, transported to the

**Table 1. Insect sampling in days after planting for the 2000 on-farm soybean variety trial.**

| On-farm research site location | | | | |
|---|---|---|---|---|
| Fontanelle | Harlan | Norway | Pella | Peosta |
| Insect sampling [Days after planting (DAP)] | | | | |
| 18 | 17 | 17 | 17 | 16 |
| 32 | 27 | 31 | 31 | 30 |
| 46 | 41 | 44 | 45 | 46 |
| 60 | 55 | 59 | 59 | 57 |
| 74 | 68 | 73 | 72 | 73 |
| 88 | 82 | 87 | 87 | 86 |
| 102 | 96 | 102 | 101 | 100 |
| — | 111 | — | 115 | 114 |
| — | — | — | 130 | — |

laboratory where they were frozen (−20°C), until samples could be sorted and identified. Soybean harvest occurred on 10 October at the Fontanelle and Harlan sites, 11 October at the Pella and Peosta sites, and 27 November at the Norway site.

**On-farm pest management trial.** An on-farm evaluation of organic-compliant treatments for bean leaf beetle and seed staining management was also initiated at the Fontenelle site in 2000. Soil samples were collected on 9 June from each 4.6 m by 9.1 m plot and analyzed for water content, electrical conductivity (EC), pH, $NH_4^-$ and $NO_3^-$-N, total organic C and N, and Mehlich III extractable P, K, Ca, Mg, and Na. Pioneer Brand 9305 soybean were planted the same day in 76-cm rows at a seeding rate of 494,000 $ha^{-1}$. Ten treatments (Table 2) were evaluated using a randomized complete block design with four replications. The rationale for choosing these treatments was based on the organic producers' belief that compost, humic acid and fulvic acid would minimize plant stress and mitigate plant disease. Baking soda and hydrogen peroxide were selected on the basis of their purported anti-fungal properties. Molasses was reported to improve plant vigor and increase resistance to bean leaf beetles. Garlic Barrier® was selected as an insect repellent, while rotenone and Neemix 4.5® were selected for direct insecticidal properties. Each plot was separated by a cultivated, 5-m border to help limit insect movement between plots. The compost and humic acid (Treatments 1 and 2) were applied once (17 June 2000), while the other treatments were applied on 17 June and repeated every other week thereafter, until 1 September 2000. Bean leaf beetle and beneficial insect sampling occurred weekly from 17 June to 1 September, following methods previously described. The plots were harvested on 10 October with a combine. The percentage of stained soybean seed was determined by counting the number of stained seeds in a 200-g sample collected from each plot.

**Experiment-station pest management trials.** Evaluations of the organic-compliant pest management treatments were continued from 2001 through 2006 at the ISU Neely-Kinyon Research Farm near Greenfield, IA. Effects of various

**Table 2. On-farm pest management treatments evaluated near Fontenelle, Iowa, in 2000.**

| Treatment No. | Material | Application rate | Supplier |
|---|---|---|---|
| 1 | Composted turkey manure | 168 kg N $ha^{-1}$ | Ultra-Gro®, Circle Hill Organics, Ellsworth, IA |
| 2 | Humic acid | 505 kg $ha^{-1}$ | Live Earth®, Emery, UT |
| 3 | Fulvic acid | 0.24 L $ha^{-1}$ | Fulvic Elctrolyte™, Enviro Consultant Service, LLC, Lakewood, CO |
| | + | | |
| | EC & S Bio-Stimulant® | 0.60 L $ha^{-1}$ | Enviro Consultant Service, LLC, Lakewood, CO |
| | + | | |
| | Liquid Compost®* | 0.24 L $ha^{-1}$ | Bachmann, Inc., West Des Moines, IA |
| 4 | Molasses§ | 14 L $ha^{-1}$ | Grandma's Molasses™, Parsippany, NJ |
| 5 | Hydrogen peroxide (3%) | 168 L $ha^{-1}$ | Equate™, Bentonville, AR |
| 6 | Baking soda§ | 5 kg $ha^{-1}$ | Arm & Hammer, Princeton, NJ |
| 7 | Rotenone | 28 kg $ha^{-1}$ | Bonide Products, Inc., Oriskany, NY |
| 8 | Garlic Barrier® | 47.2 L $ha^{-1}$ | Garlic Research Labs, Inc., Glendale, CA |
| 9 | Neemix 4.5® | 1.2 L $ha^{-1}$ | Thermo Trilogy Corp., Columbia, MD |
| 10 | Control | — | — |

*Combination mixture diluted with 74.7 L $ha^{-1}$ of water.
§Diluted with 478 L $ha^{-1}$ of water.

treatments and combinations of treatments (Table 3) on bean leaf beetle population and the incidence of seed staining were measured. The experimental design was similar to that used for the on-farm study, except that plot size was increased to 6.1 by 9.1 m and the cultivated border around each plot was also expanded to 9.1 m. The same soybean varieties were used throughout the study, with NK2412 being grown in 2001 and P9305 in all subsequent years. Surround™ (Engelhard Corp., Iselin, NJ), a kaolin clay product, was included in 2001 and 2002 because it had shown some efficacy against plum curculio [*Conotrachelus nenuphar* (Herbst)] in organic apple (*Malus domestica*) production in 2000. Planting dates were 29 May, 3 June, 28 May, 7 June, 27 May, and 23 May for 2001 through 2006, respectively. Treatments were applied every 2 week beginning 5 July, 21 June, 19 June, 6 July, 6 July, and 21 June, respectively, except for the Aphrid™ (TerraMax, Inc., Ham Lake, MN) treatment which was reported to infect soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphidae), also present in the bean leaf beetle plots. This experimental biological control treatment was applied once each year (2004 through 2006) in early August when soybean aphid populations reached >250 aphids/plant, per label recommendations. Bean leaf beetle and beneficial insect sampling occurred on alternate weeks each year from late June through September using the methods described for the on-farm study. The crop was harvested with a combine each year and the percentage of stained soybean seed was determined as previously described.

**Table 3. Experiment station pest management treatments evaluated near Greenfield, Iowa, 2001 to 2006.**

| Year | Material | Rate | Supplier |
|---|---|---|---|
| 2001 | Surround™ | 57.3 kg $ha^{-1}$ | Engelhard Corp., Iselin, NJ |
| | Garlic Barrier® | 47.2 L $ha^{-1}$ | Garlic Research Labs, Inc., Glendale, CA |
| | Neemix 4.5® | 1.2 L $ha^{-1}$ | Thermo Trilogy Corp., Columbia, MD |
| | Baking soda* | 5 kg $ha^{-1}$ | Arm & Hammer, Princeton, NJ |
| | Hydrogen peroxide (3%) | 168 L $ha^{-1}$ | Equate™, Bentonville, AR |
| | Molasses* | 14 L $ha^{-1}$ | Grandma's Molasses™, Parsippany, NJ |
| | Control | — | — |
| 2002 | Surround™ | 57.3 kg $ha^{-1}$ | Engelhard Corp., Iselin, NJ |
| | Neemix 4.5® | 1.2 L $ha^{-1}$ | Thermo Trilogy Corp., Columbia, MD |
| | Hydrogen peroxide (3%) + baking soda | 5 kg $ha^{-1}$ + 168 L $ha^{-1}$ | (see above) |
| | Garlic Barrier® + Crocker's fish oil (sticker) + molasses* | 47.2 L $ha^{-1}$ + 21 L $ha^{-1}$ + 14 L $ha^{-1}$ | (see above + Crocker's Fish Oil, Inc., Quincy, WA) |
| | Control | — | — |
| 2003 | Garlic Barrier® + Crocker's fish oil (sticker) + molasses* | 47.2 L $ha^{-1}$ + 21 L $ha^{-1}$ + 14 L $ha^{-1}$ | (see above) |
| | Neemix 4.5® | 1.2 L $ha^{-1}$ | (see above) |
| | Hydrogen peroxide (3%) + baking soda | 5 kg $ha^{-1}$ + 168 L $ha^{-1}$ | (see above) |
| | PyGanic® | 1.2 L $ha^{-1}$ | McLaughlin Gormely King Corp, Minneapolis, MN |
| | Control | — | — |
| 2004 | PyGanic® | 1.2 L $ha^{-1}$ | (see above) |
| | Neemix 4.5® | 1.2 L $ha^{-1}$ | (see above) |
| | Hexacide® | 3.5 L $ha^{-1}$ | EcoSMART Technologies, Inc., Franklin, TN |
| | Aphrid™ | 111 g $ha^{-1}$ | TerraMax, Inc., Ham Lake, MN |
| | Control | — | — |

**Table 3. Continued.**

| Year | Material | Rate | Supplier |
|---|---|---|---|
| 2005 | PyGanic® | 1.2 L $ha^{-1}$ | (see above) |
| | Hexacide® | 3.5 L $ha^{-1}$ | (see above) |
| | Entrust® | 140 g $ha^{-1}$ | Dow Agrosciences LLC, Indianapolis, IN |
| | Aphrid™ | 111 g $ha^{-1}$ | (see above) |
| | Control | — | — |
| 2006 | PyGanic® | 1.2 L $ha^{-1}$ | (see above) |
| | Hexacide® | 3.5 L $ha^{-1}$ | (see above) |
| | Entrust® | 140 g $ha^{-1}$ | (see above) |
| | Aphrid™ | 111 g $ha^{-1}$ | (see above) |
| | Control | — | — |

*Diluted with 478 L $ha^{-1}$ of water.

Grain quality analyses were conducted at the Iowa State University Grain Quality Laboratory (Ames, Iowa) from 2003 to 2006.

All data were analyzed using SAS Version 8.0 (SAS Institute, Cary, North Carolina). Least-significant differences were calculated for mean separation when the *F*-test showed significant differences among treatments at the 0.05 level of probability.

## Results

**On-farm soybean variety trial.** A significant number of bean leaf beetles were estimated to have survived through the relatively warm winter of 1999–2000 (Fig. 1). High numbers of over-wintered beetles were observed on soybean during spring 2000, especially in early-planted soybean fields. At 16 to 18 d after planting (DAP), bean leaf beetle populations ranged from 1 to 2 beetles per 20 sweeps at all sites (Table 4). Highest beetle numbers (17 to 98 beetles per 20 sweeps) were found across all sites between 55 and 74 DAP, although more than 200 beetles per 20 sweeps were present at two sites 96 to 102 DAP. The majority of soybean plants examined in this trial began senescing approximately 100 DAP, but bean leaf beetles were detected up to 130 DAP at the Pella, Iowa, site. Over the entire trial, lower beetle numbers were observed at the most northern site (Peosta) where winter temperatures were lower than at the central and southern Iowa sites.

Overall, the NK2412 soybean variety hosted fewer bean leaf beetles than the P9305 variety, with significant differences on 5 of 39 sampling dates (Table 4). The Fontanelle, Norway, and Harlan sites had significantly lower bean leaf beetle populations on NK2412 during the critical near-harvest dates approximately 102 to 111 DAP (Table 4). At the Pella site, significantly fewer beetles were observed on the NK2412 variety 17, 115, and 130 DAP. Despite the overall trend toward fewer bean leaf beetles on NK2412 at all sites, the Harlan site was the only location where NK2412 had a significantly higher yield than P9305 (Table 4). The overall average yield for NK2412 was 2.3 Mg $ha^{-1}$ compared to 2.1 Mg $ha^{-1}$ for P9305. Sites where bean leaf beetle populations were the highest (i.e., Pella and Harlan), however, did not suffer significant yield losses compared to the site with the lowest population (Peosta). Because of high variability, there were no significant differences between varieties in rates of seed staining (Table 4).

**On-farm pest management trial.** Bean leaf beetle numbers in 2000 in organic plots corresponded with populations observed across the state (Krell et al. 2004). Organic-compliant treatments, however, were not effective in lowering bean leaf beetle numbers below the control (Table 5). Soil analyses in on-farm plots showed the average water content at planting was 224 g $kg^{-1}$, with an EC of 0.44 ds $m^{-1}$, pH of 6.3, $NH_4^-$ and $NO_3^-$-N concentrations of 1.7 and 28.4 Mg $kg^{-1}$, total organic N and C levels of 3.7 and 47.3 g $kg^{-1}$, and Mehlich III extractable P and K concentrations of 167 and 346 Mg $kg^{-1}$, respectively. The EC and $NO_3^-$-N values for the compost treatment were significantly higher than the other treatments, but none of the other soil parameters were significantly different among treatments (data not presented) and soil treatments did not affect bean leaf beetle populations (Table 5).

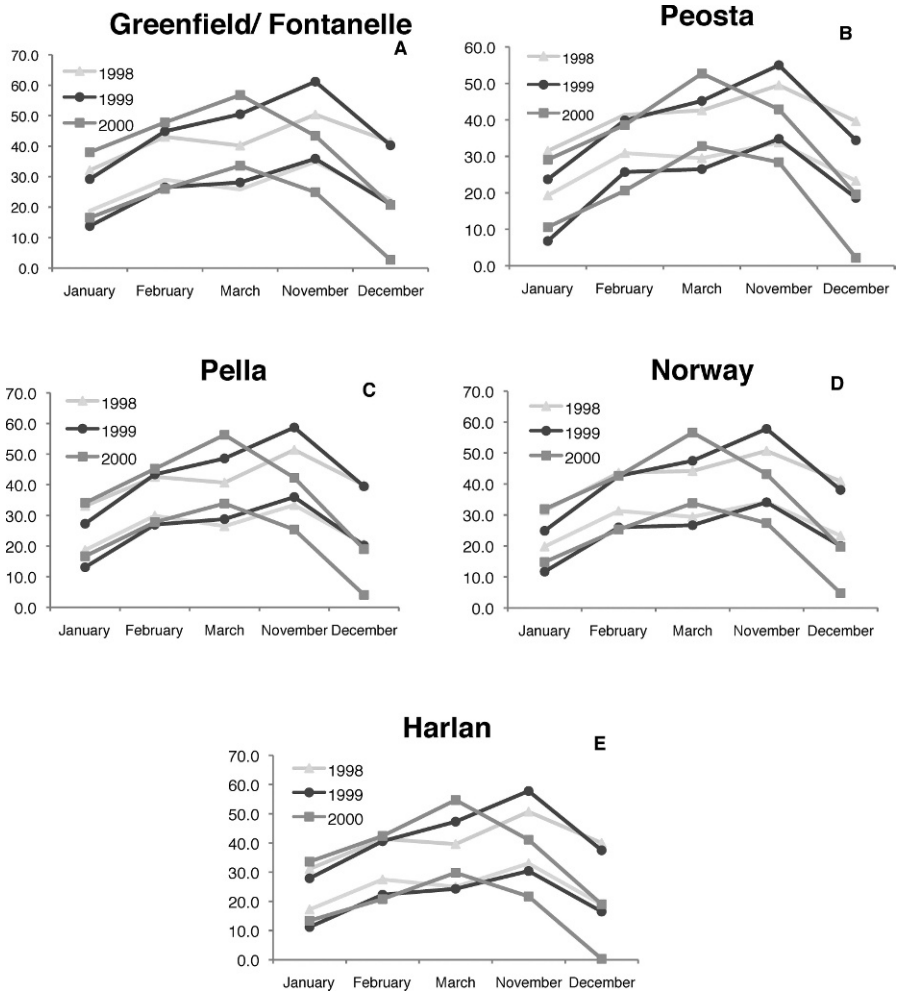


**Fig. 1.** Winter average high (top lines) and low (bottom lines) temperatures in 1998–2000 at five on-farm research locations in Iowa. Temperatures presented in °F [conversion factor: (°F – 32) * 0.556 = °C]. Source: National Climatic Data Center—National Satellite and Information Service <http://www7.ncdc.noaa.gov/IPS/cd/cd.html>. Accessed 15 July 2008.

Seasonal average populations ranged from 1 to 3 beetles per 8 sweeps, with peak populations increasing to only 4 to 7 beetles per 8 sweeps. When the bean leaf beetle population peaked during 2000 (14 July sampling date), there was a trend toward lower numbers of beetles in compost-treated plots, but the differences among treatments were not statistically significant (Table 5). Despite the low numbers of bean leaf beetles, the percentage of stained soybean seeds at harvest ranged from 15 to 20% across all treatments with no significant

**Table 4. Soybean variety effects on bean leaf beetle populations in on-farm trials throughout Iowa in 2000.**

| Farm site | Variety | Seed staining (%) | Yield (Mg ha$^{-1}$) | Bean leaf beetles collected per 20 sweeps | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 16–18 DAP | 27–32 DAP | 41–46 DAP | 55–60 DAP | 68–74 DAP | 82–88 DAP | 96–102 DAP | 111–115 DAP | 130 DAP |
| Fontanelle | Northrup King 2412 | 14.9 | 2.25 | 0.83 | 0.75 | 0.20 | 33.00 | 37.33 | 8.50 | 8.17 a | — | — |
| | Pioneer 9305 | 14.8 | 2.21 | 1.33 | 0.58 | 0.67 | 36.83 | 44.83 | 10.00 | 31.00b | — | — |
| | LSD 0.05 | ns* | ns | ns | ns | ns | ns | ns | ns | 10.35 | — | — |
| Harlan | Northrup King 2412 | 19.8 | 1.92 a | 1.25 | 1.79 | 0.21 | 44.50 | 98.67 | 148.83 | 108.17 | 78.67 a | — |
| | Pioneer 9305 | 30.8 | 1.21 b | 1.42 | 1.88 | 0.04 | 57.50 | 91.83 | 149.17 | 92.67 | 144.00 b | — |
| | LSD 0.05 | ns | 0.082 | ns | ns | ns | ns | ns | ns | ns | 16.18 | — |
| Norway | Northrup King 2412 | 18.5 | 1.52 | 0.00 | 5.25 | 46.67 | 19.67 | 28.00 | 96.00 | 25.00 a | — | — |
| | Pioneer 9305 | 28.6 | 1.37 | 0.08 | 6.58 | 59.33 | 17.33 | 23.33 | 59.67 | 59.00 b | — | — |
| | LSD 0.05 | ns | ns | ns | ns | ns | ns | ns | ns | 25.65 | — | — |
| Pella | Northrup King 2412 | 17.1 | 3.06 | 0.13 a | 0.42 | 0.17 | 35.50 | 37.33 | 24.50 | 241.83 | 42.33 a | 3.33 a |
| | Pioneer 9305 | 28.1 | 2.96 | 0.50 b | 0.46 | 0.17 | 36.00 | 34.67 | 42.00 | 323.83 | 75.17 b | 49.67 b |
| | LSD 0.05 | ns | ns | 0.35 | ns | ns | ns | ns | ns | ns | 25.11 | 18.30 |
| Peosta | Northrup King 2412 | 5.41 | 2.80 | 0.00 a | 0.56 | 0.50 | 0.25 | 7.75 | 8.00 | 1.25 | 2.00 | — |
| | Pioneer 9305 | 4.94 | 2.65 | 0.25 b | 0.69 | 1.50 | 0.00 | 9.75 | 13.25 | 2.00 | 3.75 | — |
| | LSD 0.05 | ns | ns | 0.23 | ns | ns | ns | ns | ns | ns | ns | — |

*Means with different letters in the same column each year represent significant differences, $P \leq 0.05$; ns = non-significant differences.

differences among treatments (Table 5). This percentage of stained soybean seeds, however, would downgrade the crop from tofu or food-grade quality to feed-grade soybean (Heartland Organic Marketing Cooperative, Stuart, Iowa, personal communication). With increasing demand for organic meat and milk products, feed-grade soybean would often have a premium price compared to prices for conventional soybean, but the amount was lower than that received for food-grade product. Overall, yields ranged from 1.4 to 2.1 Mg $ha^{-1}$ with no significant yield differences among treatments (Table 5). Grain quality indicators – protein, oil, fiber, carbohydrate, and moisture content – averaged 370, 188, 48, 217, and 104 g $kg^{-1}$, respectively, but did not show any differences among treatments.

**Experiment-station pest management trials.** After conducting the initial on-farm study near Fontanelle, Iowa, in 2000, effects of organic-compliant treatments on bean leaf beetle, soybean aphid, other pest insects, beneficial insects, seed staining and yield were evaluated for six additional years (2001 through 2006) at the ISU Neely-Kinyon Research Farm near Greenfield, Iowa. Although there were seasonal differences (Table 5), there were never any significant treatment effects. There were, however, some interesting trends that are summarized below.

*Beetle populations.* The winter of 2000–2001 was colder than 1999 (Fig. 1), but based on early-season insect counts (8 to 10 beetles per 20 sweeps), it appeared that a moderate number of beetles survived and resulted in greater populations than at the Fontanelle site in 2000 (Table 5). Based on reports by Lam et al. (1998), leaf litter and a relatively long-term period with snow cover presumably acted as an insulating layer and thus protected the bean leaf beetles from extreme cold. As a result, peak populations ranged from 27 to 47 beetles per 8 sweeps in 2001 compared to 4 to 6 beetles per 8 sweeps in 2000 (Table 5). In 2002, bean leaf beetle populations remained low until emergence of the first generation in mid-July. The population peaked in late July when 15 to 20 beetles were collected per 8 sweeps. Although the statewide average beetle mortality that winter was 1.5 times greater than the previous winter's mortality of 48%, the Fall 2001 population was nearly twice the size of the Fall 2000 population, which led to greater beetle numbers overall (Bradshaw et al. 2003). Once again, there were no significant differences among treatments (Table 5). The seasonal average, 5 beetles per 8 sweeps, also showed no significant differences among the treatments being evaluated (Table 5). Populations were significantly lower in 2003 than in 2002, with peak populations averaging 10 beetles per 8 sweeps, compared to 18 in 2002. The weather was significantly drier in 2003 and one result was very few beetles or signs of feeding until mid-July in 2003. Because rain is critical for moistening the soil so the adult beetles can dig their way to the surface, beetle populations were reduced in the dry summer weather (Rice 2005).

In 2004, few beetles were apparent until the end of July, which was two weeks later than in the two prior years. As a result, bean leaf beetle populations were significantly lower than in 2002 and in 2003, with the peak population averaging 6 beetles per 8 sweeps, compared with 18 in 2002 and 10 in 2003. Average beetle numbers across the entire season ranged from 1 to 3 beetles per 8 sweeps in 2004. Similarly, in 2005 very few insects were found until late July. Bean leaf beetle populations were even lower than in 2002, 2003, and 2004, with peak populations averaging 5 beetles per 8 sweeps. As a result, there were no differences in seasonal average beetle numbers (1 to 2 beetles per 8 sweeps) among treatments.

**Table 5. Insect populations, soybean staining, and yield in organic treatment trials, 2000–2006.**

| Year | Treatment | Peak beetle populations/8 sweeps | Seasonal average: Average bean leaf beetle populations/8 sweeps | Seasonal average: Average soybean aphid populations/8 sweeps | Seasonal average: Average other pest insect populations/8 sweeps* | Seasonal average: Average beneficial insect populations/8 sweeps§ | Staining (%) | Yield (Mg ha$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 2000 | Control | 4.00 | 1.70 | 0.00 | 5.47 | 0.75 | 20.43 | 1.96 |
| | Baking soda | 6.00 | 1.75 | 0.00 | 6.10 | 0.75 | 16.47 | 2.14 |
| | Composted manure | 3.75 | 1.35 | 0.00 | 4.80 | 0.85 | 18.39 | 1.37 |
| | Fulvic acid mixture | 4.50 | 1.35 | 0.05 | 4.80 | 0.65 | 17.16 | 1.90 |
| | Garlic Barrier® | 6.25 | 1.75 | 0.00 | 5.53 | 0.50 | 17.88 | 1.81 |
| | Hydrogen peroxide | 6.50 | 2.25 | 0.10 | 6.80 | 0.65 | 17.05 | 1.89 |
| | Humic acid | 6.25 | 2.00 | 0.00 | 6.55 | 0.55 | 16.64 | 1.95 |
| | Molasses | 4.50 | 1.70 | 0.00 | 5.10 | 0.40 | 15.36 | 1.98 |
| | Neemix 4.5® | 6.00 | 1.80 | 0.10 | 5.50 | 0.65 | 17.00 | 1.62 |
| | Rotenone | 5.75 | 2.10 | 0.05 | 6.50 | 0.45 | 17.88 | 1.78 |
| | LSD 0.05 | ns# | ns | ns | ns | ns | ns | ns |
| 2001 | Control | 37.33 | 15.47 | 0.07 | 25.27 | 1.00 | 22.44 | 2.77 |
| | Surround™ | 41.33 | 17.00 | 0.13 | 26.13 | 1.07 | 19.77 | 2.76 |
| | Garlic Barrier® | 34.67 | 12.40 | 0.20 | 21.47 | 1.20 | 16.21 | 2.47 |
| | Neemix 4.5® | 26.67 | 16.67 | 0.00 | 22.80 | 0.73 | 14.66 | 2.84 |
| | Baking soda | 47.00 | 16.87 | 0.07 | 23.93 | 1.67 | 18.09 | 2.69 |
| | Hydrogen peroxide | 37.33 | 14.93 | 0.07 | 24.67 | 1.40 | 12.33 | 2.62 |
| | Molasses | 45.67 | 15.33 | 0.00 | 25.87 | 1.73 | 14.84 | 2.62 |
| | LSD | ns | ns | ns | ns | ns | ns | ns |

**Table 5. Continued.**

| Year | Treatment | Peak beetle populations/8 sweeps | Seasonal average: Average bean leaf beetle populations/8 sweeps | Seasonal average: Average soybean aphid populations/8 sweeps | Seasonal average: Average other pest insect populations/8 sweeps* | Seasonal average: Average beneficial insect populations/8 sweeps§ | Staining (%) | Yield (Mg ha$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 2002 | Control | 14.50 | 5.15 | 0.00 | 9.37 | 1.67 | 7.64 | 3.70 |
| | Suround™ | 20.75 | 5.95 | 0.00 | 8.52 | 1.10 | 10.14 | 3.78 |
| | Garlic Barrier® + molasses | 19.00 | 5.05 | 0.16 | 9.44 | 1.19 | 9.88 | 3.56 |
| | Hydrogen peroxide + baking soda | 15.25 | 4.15 | 0.06 | 7.19 | 1.03 | 7.46 | 3.48 |
| | Neemix 4.5® | 18.25 | 4.60 | 0.00 | 4.37 | 1.26 | 8.61 | 3.78 |
| | LSD 0.05 | ns | ns | ns | ns | ns | ns | ns |
| 2003 | Control | 12.50 | 1.45 | — | 8.13 | 8.13 | 2.6 | 1.72 |
| | Garlic Barrier® + molasses | 13.50 | 1.64 | — | 10.38 | 6.50 | 2.7 | 1.81 |
| | Hydrogen peroxide + baking soda | 8.50 | 1.93 | — | 11.13 | 8.63 | 2.7 | 1.72 |
| | Neemix 4.5® | 8.67 | 1.66 | — | 8.13 | 7.00 | 3.0 | 1.91 |
| | PyGanic® | 8.80 | 1.57 | — | 8.63 | 4.88 | 2.4 | 1.77 |
| | LSD 0.05 | ns | ns | — | ns | ns | ns | ns |
| 2004 | Control | 5.00 | 3.26 | 1.22 | 9.04 | 2.52 | 4.20 | 3.32 |
| | Aphrid™ | 3.50 | 2.00 | 2.26 | 7.61 | 2.17 | 4.27 | 3.51 |
| | Hexacide® | 7.00 | 3.26 | 5.22 | 12.96 | 3.26 | 4.99 | 3.43 |
| | Neemix 4.5® | 3.75 | 1.78 | 2.87 | 8.70 | 2.87 | 4.60 | 3.43 |
| | PyGanic® | 8.50 | 3.00 | 4.63 | 11.88 | 3.67 | 6.72 | 3.40 |
| | LSD 0.05 | ns | ns | ns | ns | ns | ns | ns |

**Table 5. Continued.**

| Year | Treatment | Peak beetle populations/8 sweeps | Seasonal average: Average bean leaf beetle populations/8 sweeps | Seasonal average: Average soybean aphid populations/8 sweeps | Seasonal average: Average other pest insect populations/8 sweeps* | Seasonal average: Average beneficial insect populations/8 sweeps§ | Staining (%) | Yield (Mg ha$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 2005 | Control | 2.00 | 1.46 | 0.00 | 4.21 | 3.14 | 1.00 | 3.95 |
| | Hexacide® | 4.25 | 1.93 | 0.00 | 5.29 | 1.82 | 1.10 | 3.96 |
| | Entrust® | 2.00 | 1.75 | 0.00 | 5.07 | 2.93 | 1.20 | 4.06 |
| | PyGanic® | 5.25 | 1.61 | 0.00 | 4.54 | 2.32 | 1.50 | 3.89 |
| | Aphrid™ | 9.00 | 2.43 | 0.00 | 5.46 | 2.54 | 1.10 | 3.99 |
| | LSD 0.05 | ns | ns | ns | ns | ns | ns | ns |
| 2006 | Control | 17.75 | 9.05 | 2.60 | 16.10 | 4.40 | 5.19 | 3.51 |
| | PyGanic® | 14.00 | 8.10 | 1.75 | 13.90 | 3.30 | 8.08 | 3.45 |
| | Aphrid™ | 18.75 | 8.50 | 3.35 | 16.70 | 3.85 | 4.80 | 3.51 |
| | Hexacide® | 20.50 | 10.05 | 3.95 | 17.10 | 2.80 | 7.13 | 3.48 |
| | Entrust® | 15.00 | 5.10 | 3.10 | 10.80 | 4.15 | 7.42 | 3.54 |
| | LSD 0.05 | ns | ns | ns | ns | ns | ns | ns |

*Other insect pests collected included tarnished plant bug [*Lygus lineolaris* (Palisot de Beauvois)] and stink bug (*Nezara viridula* L.).

§Beneficial insects included ladybeetles [*Harmonia axyridis* (Pallas)]; *Hippodamia convergens* Guérin-Méneville; *Coleomegilla maculata* DeGeer; *Coccinella septempunctata* L.; [*Cycloneda munda* (Say)]; lacewings [*Chrysoperla carnea* (Stephens)]; damsel bugs (*Nabis* spp.); parasitic wasps (*Tetrastichus bruchophagi* Gahan); minute pirate bugs [*Orius insidiosus* (Say)]; and spiders (Thomisidae and Salticidae).

#Means with different letters in the same column each year represent significant differences, $P \leq 0.05$; ns = non-significant differences.

A similar response was noted for 2006 with the peak bean leaf beetle population ranging from 14 to 21 beetles per 8 sweeps and averaging 17 beetles per 8 sweeps in late July.

Among the treatments evaluated in 2001 (Table 3), the plots treated with Neemix 4.5® had the fewest number of beetles at peak population (6 September 2001). Throughout the entire season, there was a trend towards lower numbers of bean leaf beetles per 8 sweeps (12 to 17) in the Garlic Barrier® treatment; however, as stated previously, the differences among treatments were not significant. Differences among treatments were very small and non-significant in 2002 through 2006.

*Seed staining.* In 2001, 12 to 22% of the harvested seed was classified as being stained and would thus be rejected from the organic food-grade soybean market. The amount of seed coat staining was much lower in 2002, ranging from 7.5 to 10.1% and averaging 8.8%. There were no significant differences among the treatments (Table 5), although the lower percentage of staining did appear to be associated with lower bean leaf beetle populations. As a result of low beetle numbers, seed staining was again reduced in 2003, averaging 2.7%, with no significant difference among treatments (Table 5). In 2004, the low number of beetles resulted in a low staining percentage that averaged 3% across all treatments. Staining averaged 4% in 2005 and 7% in 2006, but differences among treatments were not significant in either year (Table 5).

*Aphids, other pest and beneficial insects.* There was no treatment effect on soybean aphid populations throughout the study. Other insect populations monitored throughout the 6-y study (Table 5) included the following: ladybeetles [*Harmonia axyridis* (Pallas)], *Hippodamia convergens* Guérin-Méneville, *Coleomegilla maculata* DeGeer, *Coccinella septempunctata* L., and *Cycloneda munda* (Say)]; lacewings [*Chrysoperla carnea* (Stephens)]; damsel bugs (*Nabis* spp.); parasitic wasps (*Tetrastichus bruchophagi* Gahan); minute pirate bugs [*Orius insidiosus* (Say)]; and spiders (Thomisidae and Salticidae) were the primary beneficial insects collected during this study. The predominant aphid species was soybean aphid. Other insect pests included tarnished plant bug [*Lygus lineolaris* (Palisot de Beauvois)] and stink bug (*Nezara viridula* L.). There were no significant treatment effects for any of these groups of insects across all years, but there was a trend showing that higher beneficial insect populations were associated with lower seed coat staining in 2002 through 2006. This trend was not apparent between beneficial insects and bean leaf beetles, possibly due to the absence of known natural enemies of the bean leaf beetle in the collected samples. The high numbers of beneficial insects appears to confirm the importance of maintaining ecological balance and may reflect the fact that the study was conducted on an organic site where pesticides were limited to this study site for several consecutive years.

*Yields.* Soybean yields in 2001 ranged from 2.5 to 2.8 Mg ha$^{-1}$ and averaged 3.7 Mg ha$^{-1}$ in 2002, with no significant differences among the organic-compliant treatments in either year. Drought resulted in much less soybean growth and eventually lower yields in 2003. Yields were not affected by beetle management practices, with control plots averaging 1.7 Mg ha$^{-1}$, compared to 1.8 Mg ha$^{-1}$ for all other treatments. Yields in 2004 were not affected by pest management techniques, with control plots averaging 3.3 Mg ha$^{-1}$ compared to an average of 3.4 Mg ha$^{-1}$ for all other treatments. Average yields for the control and treated

plots were 3.4 and 4.0 Mg ha$^{-1}$, respectively, in 2005 and averaged 3.5 Mg ha$^{-1}$ for all treatments in 2006.

*Grain quality.* In 2003, grain quality was equal across all treatments, with protein content averaging 36%. Similarly, there were no differences in grain quality among treatments for 2004, 2005 or 2006, when protein content averaged 37, 34, and 37%, respectively (data not presented).

## Discussion

While there have been some reports in the literature of lethal effects from botanical formulations, results obtained in the bean leaf beetle trials reflect the general conclusion that botanical insecticides are not as effective as synthetic formulations. A 2.5 to 5% essential oil formulation derived from *Piper aduncum* L. (Piperaceae), for example, reduced *C. tingomarianus* Bechyne adult feeding and lifespan (Fazolin et al. 2005). Scott et al. (2004) found that extracts containing isobutyl amides from three other species of *Piper* were repellent to another chrysomelid adult, the striped cucumber beetle (*Acalymma vittatum* F.) Non-target effects against ladybird beetles were detected at 0.2% concentration level, however. The use of a citrus pulp bait increased mortality of *C. trifurcata* (Harper 1981), but would need to be modified with organic-compliant material to be permitted in organic systems.

Reduced susceptibility to insect pests has been attributed to organic farming practices that build soil organic matter (Howard 1940, Phelan et al. 1995), which has been associated with moisture and mineral nutrient buffering during the growing season (Rongjun 1989, Arshad & Coen 1992). In this study, however, soil amendments did not appear to affect insect response, although organic matter was high in all sites, independent of the farming system.

Varietal differences were detected in this study, as the NK2412 soybean variety hosted fewer bean leaf beetles over the season than the Pioneer Brand 9305. These results suggest that growers should consider varietal differences based on insect pest response. Hill et al. (2006) also cited variability in BPMV symptoms and presence of the virus among soybean cultivars. Abney and Plopper (1994) noted that seed infection was significantly increased in genotypes where the virus infection delayed the rate of seed maturation.

Despite significant levels of bean leaf beetles, organic yields were economically viable in all varieties across all years. There were no yield advantages associated with use of the organic pest management treatments, however. The relationship between bean leaf beetle populations and seed staining was not as clear. All stained seed were determined to contain BPMV (data not presented), and in general, the greatest percentages of stained seed occurred in years of lower soybean yields (2000 and 2001). This corresponds with other reports showing yield reductions of up to 52% from BPMV infection (Hopkins & Mueller 1984). In 2002, when yields averaged 3.5 Mg ha$^{-1}$, seed staining was also high, at 7 to 10% (Table 5). Conversely, in 2003, when staining rates were lower (2 to 3%), yields were also low.

## Summary and Conclusions

Bean leaf beetle populations in organic fields followed those reported for conventional systems during the same time period, with 2001, 2002 and 2006

having the highest levels (Bradshaw et al. 2006). In conventional agriculture, application of synthetic insecticides against first-generation bean leaf beetles reduced BPMV incidence by 31.5% and seed coat mottling by 31.2%, compared with the unsprayed control (Krell et al. 2004). Results from our on-farm trials and seven years of experiment station research showed that organic-compliant treatments currently in use by many organic producers across the Midwest did not reduce bean leaf beetle populations compared to control plots. There were no negative impacts on beneficial insect populations.

Using a soybean price of $15 per bushel, Rice and Pope (2007) determined economical management costs for 8.1 beetles per 20 sweeps at $17.30 ha$^{-1}$ ($7 ac$^{-1}$) or $37 ha$^{-1}$ ($15 ac$^{-1}$) for 16.6 beetles. As of September 2008, organic soybean prices reached $30 per bushel (USDA-AMS 2008b), which would generate a return of $3334 ha$^{-1}$ ($1350 ac$^{-1}$) with an organic soybean yield of 3.0 Mg ha$^{-1}$ (45 bu ac$^{-1}$), signifying that yield losses up to 50% would be offset by organic premium prices. Based on these results, organic growers should continue to assess bean leaf beetle populations throughout the growing season to monitor seed coat staining, which will rise as the number of bean leaf beetles increases. They should also follow recommended practices for delayed planting and use soybean varieties showing resistance to bean leaf beetles as they are developed.

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# Bean Leaf Beetle (Coleoptera: Chrysomelidae) Response to Soybean Variety and Organic-Compliant Treatments in Iowa[1]

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**ABSTRACT** In response to concerns from certified organic producers who were experiencing significant market losses due to seed staining of soybean [*Glycine max* (L.) Merr.], we evaluated alternative ways to manage bean leaf beetles [*Cerotoma trifurcata* (Forster)] (Coleoptera: Chrysomelidae), a known vector for the seed-staining bean pod mottle virus (BPMV). From 2000 through 2006, organic-compliant treatments, including insecticidal and soil fertility products in use by organic farmers, were compared in on-farm and experiment-station trials. Two soybean varieties, Northrup-King 2412 (NK2412) and Pioneer Brand 9305 (P9305), also were evaluated for bean leaf beetle populations. Overall, the NK2412 variety hosted fewer beetles although there was not a significant yield effect. None of the organic-compliant treatments provided measurable control of bean leaf beetle populations, nor did they affect beneficial insect populations. Organic soybean yields ranged from 1.8 to 3.7 Mg $ha^{-1}$ across all years with no effect from treatments. Producers are encouraged to select soybean varieties based on insect pest response and to monitor bean leaf beetle populations to determine the effectiveness of this strategy in organic systems.

**KEY WORDS** bean leaf beetle, *Cerotoma trifurcata* Coleoptera, Chrysomelidae, soybean, *Glycine max* (L.) Merrill, seed yield, seed composition, seed staining

During the 1999 growing season, certified organic producers in Iowa requested research assistance when stained soybean [*Glycine max* (L.) Merr.] seeds were rejected from food-grade markets (e.g., tofu) at a rapidly increasing rate. Seed coat staining resulted from infection by bean leaf beetle-vectored bean pod mottle virus (BPMV), purple stain [*Cercospora kikuchii* (Mastsumoto & Tomoyasu) M.W. Gardener] and *Fusarium* spp. (Stace-Smith 1981, Lin & Hill 1983, Krell et al. 2004).

Bean leaf beetles generally have two generations in Iowa, with over-wintering adults from the previous year's second generation feeding primarily on vegetative stages of soybean (Rice & Pope 2007). Using a threshold of 7.8°C, first-generation

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# Molecular Phylogeny of Sesiidae (Lepidoptera) Inferred From Mitochondrial DNA Sequences[1]

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**ABSTRACT** Partial DNA sequence data from the mitochondrial DNA (mtDNA) cytochrome oxidase I and II genes were used to construct a molecular phylogeny based on representative species from 10 of the 20 genera of Sesiidae. Maximum likelihood, maximum parsimony, and Bayesian analysis were utilized. Sequencing of a 606-base pair region of the mtDNA cytochrome oxidase I (COI), tRNA leucine, and COII gene revealed 271 polymorphic sites among 20 species. Genetic variation ranged from 0.8 to 21.2% among species. Maximum parsimony, maximum likelihood, and Bayesian analysis do not support the recent synonmy of *Synansphecia* as *Pyropteran*. Maximum parsimony and maximum likelihood support the recent divergence of *Synanthedon pamphyla* from *Synanthedon culciformis,* which are almost identical morphologicaly. Maximum likelihood, parsimony, and Bayesian analysis do not support the inclusion of *Melittia cucurbitae* in the Sesiinae subfamily. All analysis support *Synanthedon* included in the Sesiinae subfamily. All analysis also give support for *Vitacea* and *Paranthrene* forming the subfamily Paranthrenini. This is the first attempt to resolve relationships within Sesiidae with molecular data. Sesiidae are a divergent order of Lepidoptera in which many relationships should be examined more closely. Future studies should investigate nucleur markers to further support relationships supported by molecular data.

**KEY WORDS** COI, COII, 18S, Sesiidae, Lepidoptera

Larvae of many species of Sesiidae, the clearwing moths, are important pests in commercial nurseries, urban landscapes, timber stands, vineyards, and orchards (Nielson 1978). They cause economic loss by larval boring in stems and roots of herbaceous and woody plants. Most species are univoltine (requiring one year for development), but some require more than one year to develop. Sesiid species in the genera *Podosesia* Möschler, *Paranthrene* Hübner and *Synanthedon* Hübner cause economic loss to commercial nurseries and timber producers in the United States (Solomon et al. 1982). If not controlled, the peachtree borer, *Synanthedon exitiosa* (Say), and the lesser peachtree borer, *S. pictipes* (Grote and Robinson) can destroy entire orchards of fruit trees. Several species in the genera *Vitacea* Engelhardt, *Melittia* Hübner and *Pennisetia* Dehne can cause serious losses to various crops (Solomon & Dix 1979).

Morphological classification of Sesiidae has undergone several revisions since the 1960s (Naumann 1971, Bradley et al. 1972, Bradley & Fletcher 1974,

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Duckworth & Eichlin 1974, 1977, Hepner & Duckworth 1981). Naumann (1971) proposed two subfamilies: Tinthiinae with the tribes Tinthiini and Pennisetiini and Sesiinae with the tribes Sesiini, Melittiini, Paranthrenini and Aegeriini. Bradley et al. (1972) and Bradley & Fletcher (1974) proposed a third subfamily, Paranthreninae, which holds tribes, Paranthrenini and Synanthedonini. Duckworth & Eichlin (1974, 1977), Heppner & Duckworth (1981), and Eichlin & Duckworth (1988) agree that the subfamily Paranthreninae should be recognized, but without the tribe Synanthedonini, which is placed in the subfamily Sesiinae. Lastuvka & Lastuvka (2001) argue that the anagenetic changes in the tribe Paranthrenini are not distinct enough to require the establishment of a separate subfamily and follow Naumann's (1971) classification including only two subfamilies.

Eichlin & Duckworth (1988) placed 86 of the 123 described species of Sesiidae from America north of Mexico in the Synanthedonini, which accounts for 70% of the fauna. The 41 species of *Synanthedon* are morphologically grouped on the basis of similarities in genitalia. The subgroupings of *Synanthedon* correspond to several genera that were recognized by previous workers (Engelhardt 1946, Naumann 1971). Duckworth & Eichlin (1977) were convinced that these taxa had no concordance with other sesiid genera and were only defined by a few genetalic features. These character states often overlap from one taxon to another, so these genera were placed under *Synanthedon*. A molecular phylogenetic analysis could give insight among relationships within this family and should especially focus on the genus *Synanthedon*.

Like many Lepidoptera, Sesiidae use sex pheromones released by the female. Several species can be attracted to the same sex attractant (Payne et al. 1973), and cross-attraction sometimes occurs between males and females of different species (Comeau & Roelofs 1973). In cases where pheromone differences are not premating isolation mechanisms, other mechanisms exist, such as adult emergence on alternate years or different times of season, mating at different vegetational hosts or strata and geographic seperation (Sanders 1971, Brown 1972). Knowledge of the genetic relationships of sesiid species may help clarify the observed specificity of sex pheromones and observed behaviors.

In order to resolve difficulties in the classification of the family based on morphology, we tested molecular techniques to construct phylogenetic hypotheses based on DNA markers. The mitochondrial cytochrome oxidase I and II genes have been used extensively to infer phylogenetic relationships in insect families such as Drosophilidae (Simon et al. 1994), Tephritidae (Smith et al. 2003), Rhinotermatidae (Austin et al. 2004) and various families of Lepidoptera (Brower 1994, Sperling & Hickey 1994, Landry et al. 1999, Lange et al. 2004) and might be useful and appropriate for phylogenetic reconstruction of the lepidopteran family Sesiidae. Animal mitochondrial genes are known to evolve more rapidly than nuclear genes and are therefore good markers to analyze relatively close relationships, such as species relationships within a genus.

The phylogenetic relationships among members of Sesiidae and the amount of genetic variation among species were determined by 3 techniques using: the DNA sequences of the COI and COII genes; and mitochondrial DNA (mtDNA) sequence data. These data resulted in the formulation of a hypothesis of relationships and evolutionary history among genera and species of the family Sesiidae.

## Materials and Methods

Sesiids were collected from four locations in Arkansas: Carrol Co., Faulkner Co., Madison Co., and Washington Co. during 2005 (Table 1). The moths were caught using commercially available pheromone lures placed in Trécé Pherocon IC wing traps (Trécé Inc., Adair, OK). The following lures were used for sesiid capture (abbreviations refer to lure type): oak borer (OB), *Paranthrene simulans* (Grote) and lilac borer (LCB), *Podosesia syringae* (Harris) lures from Scentry (Billings, MT); dogwood borer (DWB), *Synanthedon scitula* (Harris); lilac borer (LB); grape root borer (GRB), *Vitacea polistiformis* (Harris); lesser peachtree

**Table 1. Sesiidae collection data including: species, sample number, and collection site.**

| Species name | Sample or accession # | Collection site city, county, state, country |
|---|---|---|
| *Pennisetia marginata* | 1, 150, 148, 149 | Conway, Faulkner Co., AR, USA |
| *Vitacea polistiformis* | 131, 134 | Conway, Faulkner Co., AR, USA |
| *Paranthrene simulans* | 4, 5, 6 | Fayetteville, Washington Co., AR, USA |
| *P. simulans* | 14, 15 | Berryville, Carroll Co., AR, USA |
| *P. simulans* | 19 | Conway, Faulkner Co., AR, USA |
| *P. simulans* | 43 | Fayetteville, Washington Co., AR, USA |
| *P. simulans* | 79 | Hindsville, Madison Co., AR, USA |
| *P. simulans* | 99 | Conway, Faulkner Co., AR, USA |
| *Synanthedon pictipes* | 10, 11 | Berryville, Carroll Co., AR, USA |
| *S. pictipes* | 46, 87 | Fayetteville, Washington Co., AR, USA |
| *S. exitiosa* | 22, 73 | Conway, Faulkner Co., AR, USA |
| *S. scitula* | 25, 37 | Fayetteville, Washington Co., AR, USA |
| *S. scitula* | 49, 50 | Berryville, Carroll Co., AR, USA |
| *S. rileyana* | 36, 128 | Fayetteville, Washington Co., AR, USA |
| *S. rileyana* | 56, 57 | Berryville, Carroll Co., AR, USA |
| *S. rileyana* | 64 | Conway, Faulkner Co., AR, USA |
| *S. culiciformis* | AY304170 | Russia |
| *S. culiciformis* | AY304168 | Germany |
| *S. pamphyla* | AY304169 | Turkey |
| *S. spheciformis* | AJ862900 | Austria |
| *Podosesia syringae* | 29 | Conway, Faulkner Co., AR, USA |
| *P. syringae* | 127 | Conway, Faulkner Co., AR, USA |
| *Melittia cucurbitae* | 32, 33 | Fayetteville, Washington Co., AR, USA |
| *M. cucurbitae* | 68, 69 | Conway, Faulkner Co., AR, USA |
| *M. cucurbitae* | 77, 81 | Hindsville, Madison Co., AR, USA |
| *Chamaesphecia tenthrediniformis* | AJ862898 | Spain |
| *Bembecia ichneumoniformis* | AJ862897 | Austria |
| *B. uroceriformis* | AJ862893 | Greece |
| *B. psoraleae* | AJ862898 | Spain |
| *B. lomatiaeformis* | AJ862899 | Greece |
| *Pyropteron chrysidiforme* | AJ862901 | Italy |
| *P. minianiforme* | AJ862902 | Greece |
| *Synansphecia kautzi* | AJ862903 | Spain |

borer (LPTB), *Synanthedon pictipes* and greater peachtree borer (GPTB), *Synanthedon exitiosa* lures from Trécé Inc.; raspberry clear-wing borer (RCW), *Pennisetia hylaeiformis* (Laspeyres) and squash vine borer (SVB), *Melittia cucurbitae* lures from Pherobank (Wageningen, Netherlands); and raspberry crown borer (RCB), *Pennisetia marginata* (Harris) lure from IPM Tech. (Portland, OR).

Seven traps were located at the University of Arkansas Experiment Station (Fayetteville, Washington County, AR) with the following lures: OB, LCB, DWB, LB, RCW, GRB, SVB, and RCB. Five traps baited singly with the following lures were located at a commercial apple and peach orchard in Berryville, Carroll County, AR: LB, DWB, RCW, LPTB and GPTB. Nine traps baited singly with the following lures were located at a commercial apple, peach, and blackberry orchard in Conway, Faulkner County, AR: LCB, DWB, LB, RCW, GRB, SVB, RCB, GPTB, and LPTB. Two GRB lure baited traps were located at a commercial vineyard in Hindsville, Madison County, AR. Traps were placed in the field in May and checked weekly through September. After specimens were collected from traps, they were identified using morphological keys (Eichlin & Duckworth 1988), and stored in glass specimen tubes at −20°C until DNA extraction. Voucher specimens are deposited in the University of Arkansas Arthropod Museum, Fayetteville, AR.

DNA was extracted from the thoraces of individual specimens using the Puregene® DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μL of Tris: EDTA (10 mm Tris-HCl, 1 mm EDTA, pH = 8.0) and stored at −20°C. Mitochondrial DNA PCR was conducted using primers C1-J-2797 (5′-CCTCGACGTTATTCAGATTACC-3′) (Simon et al. 1994) and C2-N-3400 (5′-TCAATATCATTGATGACCAAT-3′) (Taylor et al. 1997). These primers amplify approximately 606 bp of the mtDNA cytochrome oxidase I gene (COI), tRNA-leu and cytochrome oxidase II gene (COII). PCR reactions were conducted using 2 μL of the extracted DNA. The thermal cycler profile for this region of mtDNA gene consisted of 35 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 45 s per Szalanski et al. (2000). Excess dNTP's and primers were removed and the amplified DNA concentrated using minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega). Samples were sent to the University of Arkansas Medical School Sequencing Facility (Little Rock, AR) for direct sequencing in both directions. Sequence data were deposited in GenBank accession numbers DQ205539 to DQ205573.

The sesiid mitochondrial COI/COII sequences were initially aligned using Clustal W (Thompson et al. 1994) and subsequently refined by eye using BioEdit 5.89 (Hall 1999). Only 606 bp unambiguously aligned positions were used for analyses. The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura 1980) of sequence evolution. Mitochondrial DNA sequences from the following sesiids were added from GenBank (Table 1): *Synanthedon culiciformis* (Linnaeus), *S. pamphyla* (Kallies), *Chamaesphecia tenthrediniformis* (Denis and Schiffermüller), *Bembecia ichneumoniformis* (Denis and Schiffermüller), *B. uroceriformis* (Treitschke), *B. psoraleae* (Bartsch and Bettag), *B. lomatiaeformis* (Lederer), *Pyropteron chrysidiforme* (Esper), *P. minianiforme* (Freyer), *Synansphecia kautzi* (Reisser), and *Synanthedon spheciformis* (Denis and Schiffermüller). DNA sequences were aligned using Clustal W (Thompson et al. 1994).

For model based phylogenetic analyses (i.e., Maximum Likelihood, Bayes) the best-fitting nucleotide substitution model was chosen according to the GTR+Γ model as selected from 64 different models using ModelTest v 3.7 (Posada & Crandall 1998) and PAUP* 4.0b10 (Swofford 2001). Phylogenetic analyses was conducted with maximum likelihood (ML) analysis using the best-fitting evolutionary model in PAUP*. Maximum likelihood bootstrapping was performed using stepwise addition (1000 replicates) to determine the reliability of obtained topologies. Phylogenetic trees were also obtained using Bayesian inference with the GTR+Γ model using MrBayes. There were 2 million generations with trees saved every 100 generations, and the split frequency distribution value used as a test for convergence of parameters was 25% to determine the number of trees discarded as burnin (5000). Unweighted parsimony (MP) analyses on the alignments were conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as missing data and 11 random addition sequences was used. A bootstrap test was used to test the reliability of trees (Felsenstein 1985). Parsimony bootstrap analysis included 1000 resamplings by using stepwise addition PAUP*. *Proserpinus clarkiae* (Boisduval) (Lepidoptera: Sphingidae), was used as the outgroup taxon.

## Results

DNA sequencing of the mtDNA amplicon resulted in an average amplicon size of 606 bp. Nucleotide positions 1–220 were COI, 221–288 tRNA-leu, and 289–606 were COII. The aligned data matrix, including the outgroup taxon resulted in a total of 653 characters. Of these, 382 (59%) were fixed, 40 (6%) were phylogenetically uninformative, and 231 (35%) were phylogenetically informative.

The data set produced one most-parsimonious tree (Fig. 1) length = 811, CI = 0.446 as documented using a heuristic search in PAUP*, with 4 distinct groups. Maximum likelihood analysis recovered an optimal ML tree −ln likelihood = 6032 with nucleotide frequencies of A = 40%, C = 10%, G = 4% and T = 47%. Bootstrap ML analysis of the aligned sesiids and the outgroup taxon resulted in a consensus tree with many branches supported by values >50. The ML tree resolved four distinct groups similar to those found with the MP analysis. Bayesian analysis of the dataset also converged on four groups, although group 3 changed positions to share a node with group 1 (Fig. 2).

Pairwise Tajima Nei distances (Tajima & Nei 1984) within Sesiidae for mtDNA sequences ranged from 0.8% between *Synanthedon pamphyla* and *S. culiciformis* to 20.9% between *Pennisetia marginata* and *S. culiciformis* (Table 2). Within *Synanthedon*, genetic variation ranges from 0.8% between *S. pamphyla* and *S. culiciformis* to 11.9% between *S. rileyana* and *S. spheciformis*. Divergence between *P. marginata* and all other genera ranged between 14.1% (*Paranthrene simulans* and *Melittia cucurbitae*) to 21.2% (*Synanthedon spheciformis*). Divergence among *Bembecia* genera and other sesiid genera ranged from 8.9% (*Synansphecia kautzi*) to 19.8% (*Vitacea polistiformis*). *Vitacea polistiformis* divergence when compared to other sesiid genera ranged from 7.1% (*P. simulans*) to 19.8% (*Bembecia psoraleae*). *Melittia Cucurbitae* divergence when compared to other sesiid genera ranged from 12.5% (*P. simulans*) to 16.3% (*Synanthedon culiciformis*). *Chamaesphecia tenthrediniformis* divergence compared to other

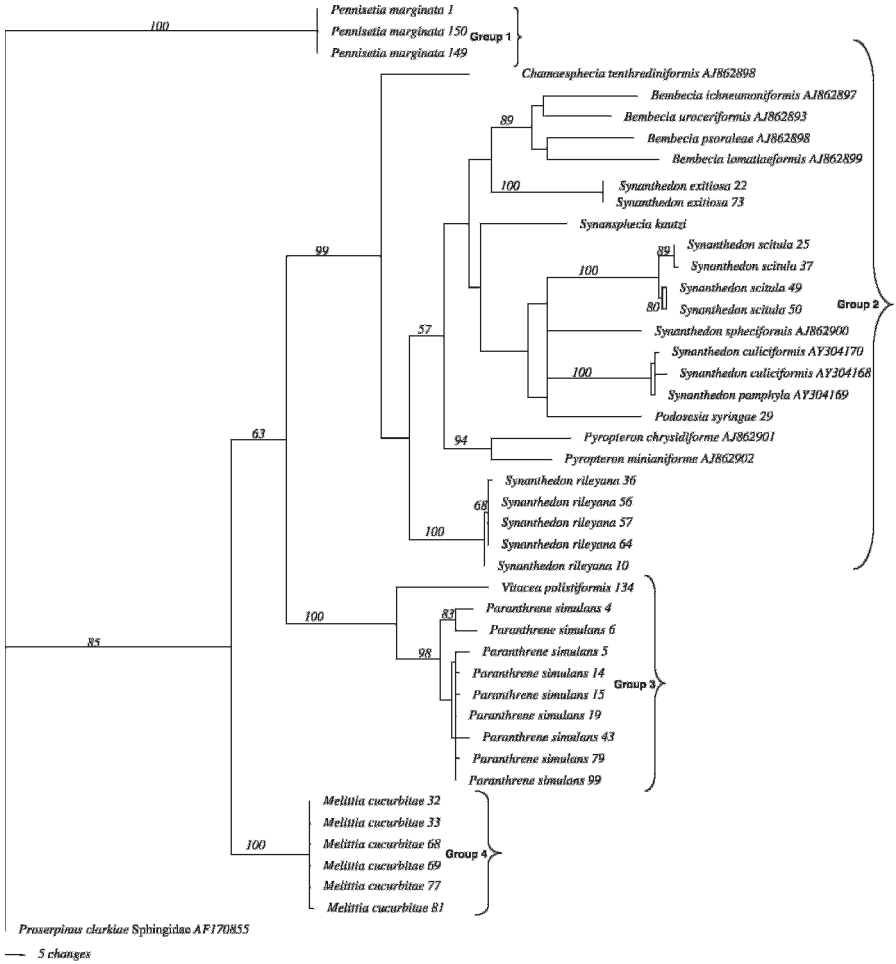


**Fig. 1.** Maximum parsimony phylogram of 10 genera of Sesiidae. Bootstrap values for 1000 replicates are listed above the branches supported at ≥50%.

sesiid genera ranged from 9.1% (*Synanthedon rileyana*) to 17.6% (*Pennisetia marginata*). *Pyropteron* divergence ranged from 7.9% (*Synansphecia kautzi*) to 19.2% (*P. marginata*) when compared to other genera of Sesiidae. *Synansphecia kautzi*'s divergence when compared to other sesiid genera ranged from 7.9% (*P. chrysidiforme*) to 18.2% (*P. marginata*). Pairwise Tajima Nei distances compared among Sesiids and the outgroup taxon *Proserpinus* ranged from 13.6 to 19.3%.

## Discussion

This study represents the first attempt to address the phylogenetic relationships within the clearwing moth family Sesiidae at the molecular level. Most of the inferred relationships had strong quantitative support as determined by bootstrap analyses (Figs. 1 and 2). The relationships among genera inferred

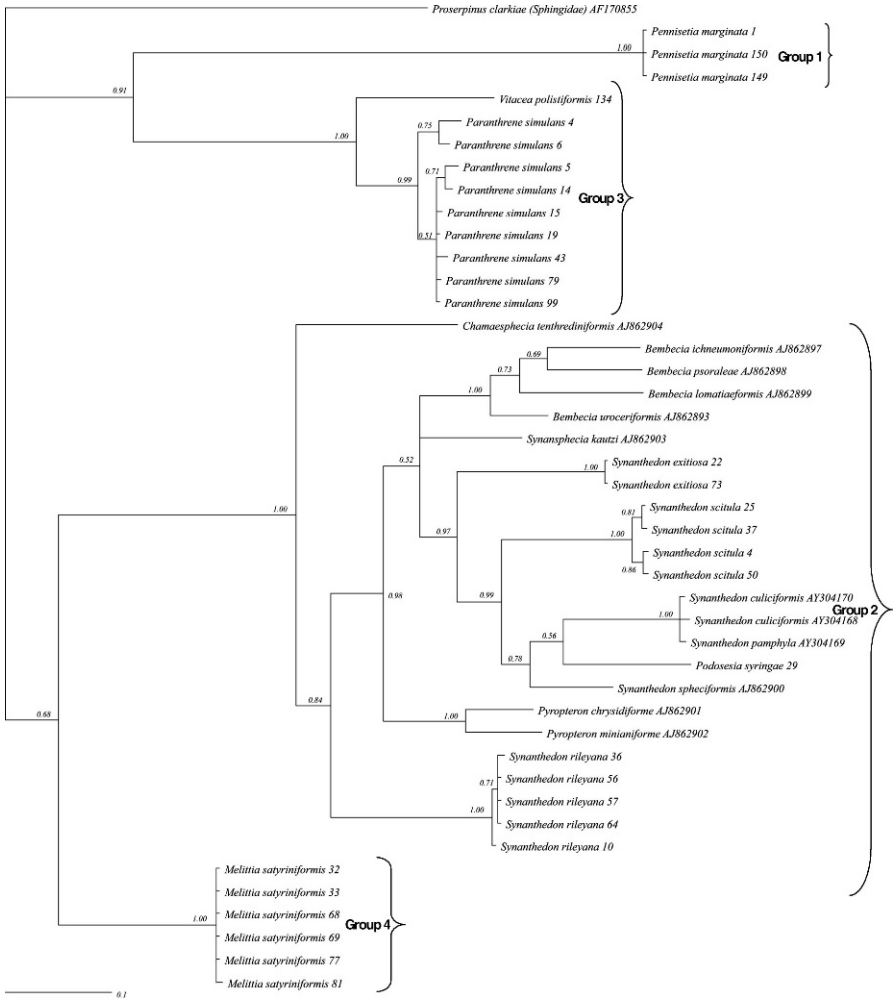


**Fig. 2.** Phylogram obtained by Bayesian analysis for 10 genera of Sesiidae. Posterior bootstrap values are listed above the branches supported at ≥50%.

from maximum parsimony and maximum likelihood analysis raised many questions about relationships among Sesiidae and gave support for many morphologicaly established relationships.

Four groups were resolved within Sesiidae. Group 1 consisted of sequences from a single species *Pennisettia marginata*. There was a clear delimination between group 1 and all other groups supported by MP, ML, and Bayesian analysis (Figs. 1 and 2). Maximum parsimony analysis did not include this group in monophyletically with the other groups (Fig. 1). This group is the most divergent compared to the other groups and its branching basal to other sesiids may be do to long branch attraction artifact (where the most divergent sequences tend to branch together) (Lartillot et al. 2007). Sequences from other *Pennisettia*

**Table 2. Tajima-Nei pairwise distances within and among 20 species of Sesiidae.**

| Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 *P. marginata* | 0.3 | | | | | | | | | | | | | | | | | | | |
| 2 *C. tenthrediniformis* | 17.6 | – | | | | | | | | | | | | | | | | | | |
| 3 *B. ichneumoniformis* | 19.7 | 11.6 | – | | | | | | | | | | | | | | | | | |
| 4 *B. uroceriformis* | 19.6 | 11.4 | 7.5 | – | | | | | | | | | | | | | | | | |
| 5 *B. psoraleae* | 19.7 | 13.6 | 8.2 | 8.0 | – | | | | | | | | | | | | | | | |
| 6 *B. lomatiaeformis* | 18.7 | 12.3 | 9.5 | 8.8 | 10.0 | – | | | | | | | | | | | | | | |
| 7 *P. chrysidiforme* | 19.0 | 11.6 | 10.0 | 10.1 | 12.5 | 12.6 | – | | | | | | | | | | | | | |
| 8 *P. minianiforme* | 19.2 | 11.1 | 11.6 | 9.4 | 12.0 | 12.7 | 6.9 | – | | | | | | | | | | | | |
| 9 *S. kautzi* | 18.2 | 10.5 | 9.3 | 8.9 | 9.6 | 11.5 | 7.9 | 8.8 | – | | | | | | | | | | | |
| 10 *V. polistiformis* | 16.4 | 16.3 | 18.0 | 18.0 | 19.8 | 19.1 | 15.1 | 16.3 | 16.6 | – | | | | | | | | | | |
| 11 *P. simulans* | 14.1 | 14.8 | 17.6 | 18.1 | 19.4 | 18.3 | 16.3 | 16.5 | 16.7 | 7.1 | 1.4 | | | | | | | | | |
| 12 *S. pictipes* | 19.0 | 10.0 | 10.4 | 10.3 | 12.4 | 11.6 | 10.1 | 10.8 | 9.2 | 16.8 | 16.5 | 3.5 | | | | | | | | |
| 13 *S. exitiosa* | 17.7 | 10.1 | 11.2 | 10.3 | 11.7 | 11.7 | 11.1 | 11.1 | 10.0 | 17.8 | 16.3 | 8.0 | 0.0 | | | | | | | |
| 14 *S. scitula* | 19.5 | 16.2 | 12.0 | 12.1 | 12.8 | 13.2 | 11.9 | 12.3 | 10.5 | 17.8 | 18.3 | 9.1 | 10.8 | 0.8 | | | | | | |
| 15 *S. rileyana* | 16.5 | 9.1 | 11.6 | 10.2 | 12.1 | 11.9 | 10.1 | 10.1 | 9.4 | 15.1 | 14.3 | 8.8 | 11.0 | 11.3 | 0.2 | | | | | |
| 16 *S. culiciformis* | 20.9 | 10.6 | 12.0 | 10.8 | 12.2 | 14.8 | 11.3 | 12.8 | 9.7 | 18.8 | 18.8 | 10.7 | 10.3 | 11.1 | 10.9 | 0.9 | | | | |
| 17 *S. pamphyla* | 20.7 | 10.9 | 11.9 | 10.7 | 11.8 | 14.4 | 11.3 | 12.7 | 9.5 | 18.6 | 18.7 | 10.6 | 9.9 | 11.2 | 10.8 | 0.8 | – | | | |
| 18 *S. spheciformis* | 21.2 | 12.1 | 11.9 | 11.3 | 12.4 | 13.3 | 12.4 | 11.6 | 11.2 | 18.5 | 18.7 | 9.1 | 9.1 | 10.3 | 11.9 | 9.4 | 9.4 | – | | |
| 19 *P. syringae* | 18.8 | 10.7 | 11.6 | 10.9 | 12.3 | 12.6 | 10.6 | 9.9 | 9.7 | 17.1 | 17.0 | 8.4 | 9.3 | 9.7 | 9.8 | 10.4 | 10.1 | 9.8 | – | |
| 20 *M. cucurbitae* | 14.1 | 12.8 | 14.8 | 14.2 | 16.6 | 15.3 | 14.2 | 14.1 | 14.3 | 13.7 | 12.5 | 13.3 | 13.6 | 14.1 | 12.6 | 16.3 | 16.2 | 15.0 | 14.1 | 0.1 |

species should be added in the future, which may break the long-branch leading to this taxon and help get rid of possible long-branch attraction artifact. This divergence is also supported by pair-wise differences with divergence between *P. marginata* and all other genera ranging between 14.1% (*P. simulans* and *M. cucurbitae*) to 21.2% (*S. spheciformis*) (Table 2).

*Pennisettia marginata* has a narrow host range feeding on only *Rubus* species. *Rubus* is an ancient group of plants with fossils dating back to the Eocene (55.8–33.9 million years ago) (Devore & Pigg 2006). Since the host plants are quite ancient, it is not far fetched to believe that *P. marginata* could be a more historic genus than others in Sesiidae. A small amount of variation was found among the three *P. marginata* specimens with 0.3% divergence.

Group 2 included all genera grouped in the subfamily Sesiinae. This group was robustly supported in MP, ML, and Bayesian analysis (Figs. 1 and 2). Some relationships supported by high bootsrap values conflict with current morphological classification. Lastuvka & Lastuvka (2001) synonymized *Pyropteran* and *Synansphecia* because no clear distinguishing morphological characteristics can be found between these two genera. In all optimal trees *Synansphecia* and *Pyropteron* never branch together. It appears unlikely that they share a most recent common ancestor exclusive of all other taxa. Calculations of pair-wise genetic divergence between *S. kautzi* and *P. chrysidiforme* is 7.9% divergent, and *S. kautzi* and *P. minianiforme* is 8.8% divergent which gives support to the findings.

Group 2 is monophyletic and strongly supported and composed of 6 genera. This was reconstructed in MP, ML, and Bayesian analysis (Figs. 1 and 2). It is interesting to note that all *Chamaesphecia* species utilize a unique host plants in the *Euphorbia* genus (Lastuvka & Lastuvka 2001). No other sesiids represented in the data set feed on these host plants. Many of these plants have a sap that is very toxic to herbivores. This association could have caused coevolution of *C. tenthrediniformis* with its host plant, which could convolute phylogenetic analysis. From all trees *C. tenthrediniformis* is the most evolutionary divergent from all other taxa in group 2.

In group 2, the MP, ML, and Bayesian analysis did not resolve the relationship of *S. pamphyla* and *S. culiciformis* (Figs. 1 and 2). Divergence data supports that *S. pamphyla* is probably a synonym of *S. culiciformis* with only 0.8% divergence between the species (Table 2). If *S. pamphyla* is a distinct species within the *Synanthedon* genus it has less divergence among sister species than any other genera in this study, which is unlikely due to the large amount of genetic variation shown between all other species in the same genus. The two *S. culiciformis* sequences were found to be 0.9% divergent, which is slightly greater than 0.8% divergent when *S. culiciformis* is compared to *S. pamphyla*. Kallies (2003) found divergence between *S. pamphyla* and *S. culiciformis* to range between 0.8–1% using mtDNA.

*Synanthedon culiciformis* has been described as very similar to *S. pamphyla* with very similar genitalia. Although, external morphological differences have been found to exist with *S. pamphyla* having: a broader discal spot, smaller ETA of forewing, broader apical area, opaque cell between Cu1 and Cu2, absence of red scales at the forewing base, black labial palps, black legs, a different color of the abdomen, and larger size (Kallies 2003). Kallies (2003) applied a molecular clock to find out the corresponding age of the separation of *S. culiciformis* and *S.*

*pamphyla*, which was estimated at 300 to 500,000 y, which may explain the small number in divergence and unresolved molecular phylogeny.

Group 3 includes *Vitacea polistiformis* and *Paranthrene simulans*, which are both grouped within the subfamily Paranthreninae first established by Bradley et al. (1972) then modified by Duckworth & Eichlin (1974, 1977) and Heppner & Duckworth (1981). This relationship is supported by MP, ML, and Bayesian analysis (Figs. 1 and 2).

The position of group 3 varied depending on the method of phylogenetic analyses. The Paranthreninae are embedded in a clade with groups 2 and 4 in MP and ML trees while they strongly branch with *Pennisetia* (group 1), to the exclusion of groups 2 and 3 in Bayesian analyses. These branchings of MP and ML are supported by a morphologically-based classification scheme proposed by Lastuvka & Lastuvka (2001) (Fig. 1). Bayesian analysis supports the classification schemes proposed by Heppner, Eichlin & Duckworth (1988) in the establishment of a third subfamily Paranthreninae (Fig. 2). When divergence is considered *P. simulans* and *V. polistiformis* have a large amount of divergence compared to all other species in the other two subfamilies. This also gives support to the Paranthreninae subfamily.

Group 4 is represented by the single species *Melittia cucurbitae*, which is the only member of the tribe Melittiini in the subfamily Sesiinae (Figs. 1 and 2). Analysis of MP, ML, and Bayesian did not group *M. cucurbitae* within the other genera of the Sesiinae recovered in group 2 of all the analyses. This group branched basal to all other Sesiinae genera and may represent an early divergence. This species utilizes host plants in the family Cucurbitaceae, unlike other Sesiinae genera. In the subfamily Sesiinae, divergence ranged from12.5% (*M. cucurbitae*) to 19.8% (*B. psoraleae*). This is the greatest amount of divergence between tribes within the same subfamily thus supporting an earlier divergence.

*Vitacea* (group 3) and *Melittia* (group 2) share a node in the MP and ML analyses. Interestingly, these species respond to the same sex pheromones, (2E, 13Z)-2,13-octadecadien-1-ol acetate and (3Z, 13Z)-3,13-octadecadien-1-ol (Klun et al. 1990, Schwarz et al. 1983). This is supportive evidence that a common ancestor was recently shared. The divergence between these two species is 12.5%, which is the least amount of divergence between *Vitacea* and any other member outside of the Paranthrenini tribe (Table 2). The node shared by *Vitacea* and *Melittia* in MP and ML is also shared by the rest of the group 2 taxa, but the ancestral state could be a response to this pheromone with multiple pheromone switching in groups 3 and 4.

This study shows that Sesiidae have a large amount of genetic variation among species. Within *Synanthedon*, divergence ranges from 0.8–11.9% (Table 2). Landry et al. (1999) found that divergence among certain *Argyrotenia* species (Lepidoptera: Tortricidae), using the mitochondrial oxidase II gene, ranges from 2.6 to 9.3%. Sesiid divergence is slightly greater. Genetic divergence within *Coptotermes* termite species (Isoptera: Rhinotermitidae) ranges from 0.0–8.0% (Austin et al. 2004). Divergence within Sesiidae ranges from 0.8% between *S. pamphyla* and *S. culiciformis* to 21.2% between *P. marginata* and *S. spheciformis* (Table 2).

Host plant specificity could be leading to some of the variation and divergence found. Mitter & Futyuma (1978) studied the genetic consequences of feeding habits of some forest dwelling Lepidoptera and found that specialized feeders

(feeding on one family of host plants) have more genetic variation than generalized feeders (feeding on two or more families of host plants). Specialists could accumulate genetic variation due to local variation or lower migration rates in between environmental patches (Mitter & Futuyuma 1978).

Generalists could have a "homeostatic" mechanism that reduces the environmental variation perceived by loci (Mitter & Futuyuma 1978). If specialized species lacked this mechanism chemical changes and differences among host plants could maintain genetic variation that would not be seen in more generalized species. This hypothesis could account for the amount of genetic variation observed among sesiids.

Although many currently accepted morphologically-based classifications have been supported by our results, some taxa represented in this data set prompt as many questions as they answer. Sequence data from more genera and species of the Sesiinae subfamily, should be evaluated to strengthen or weaken molecular relationships. Future research should focus on resolving relationships within the entire family, and finding a nuclear marker or informative microsattellite loci. This study brings to light a wealth of interesting relationships to be studied within the group, especially those of each species with its host plant. A future study of mapping the host plant phylogenies on the sesiid phylogenies would be an interesting way to interpret relationships.

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# Molecular Phylogeny of Sesiidae (Lepidoptera) Inferred From Mitochondrial DNA Sequences[1]

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**ABSTRACT** Partial DNA sequence data from the mitochondrial DNA (mtDNA) cytochrome oxidase I and II genes were used to construct a molecular phylogeny based on representative species from 10 of the 20 genera of Sesiidae. Maximum likelihood, maximum parsimony, and Bayesian analysis were utilized. Sequencing of a 606-base pair region of the mtDNA cytochrome oxidase I (COI), tRNA leucine, and COII gene revealed 271 polymorphic sites among 20 species. Genetic variation ranged from 0.8 to 21.2% among species. Maximum parsimony, maximum likelihood, and Bayesian analysis do not support the recent synonmy of *Synansphecia* as *Pyropteran*. Maximum parsimony and maximum likelihood support the recent divergence of *Synanthedon pamphyla* from *Synanthedon culciformis,* which are almost identical morphologicaly. Maximum likelihood, parsimony, and Bayesian analysis do not support the inclusion of *Melittia cucurbitae* in the Sesiinae subfamily. All analysis support *Synanthedon* included in the Sesiinae subfamily. All analysis also give support for *Vitacea* and *Paranthrene* forming the subfamily Paranthrenini. This is the first attempt to resolve relationships within Sesiidae with molecular data. Sesiidae are a divergent order of Lepidoptera in which many relationships should be examined more closely. Future studies should investigate nucleur markers to further support relationships supported by molecular data.

**KEY WORDS** COI, COII, 18S, Sesiidae, Lepidoptera

Larvae of many species of Sesiidae, the clearwing moths, are important pests in commercial nurseries, urban landscapes, timber stands, vineyards, and orchards (Nielson 1978). They cause economic loss by larval boring in stems and roots of herbaceous and woody plants. Most species are univoltine (requiring one year for development), but some require more than one year to develop. Sesiid species in the genera *Podosesia* Möschler, *Paranthrene* Hübner and *Synanthedon* Hübner cause economic loss to commercial nurseries and timber producers in the United States (Solomon et al. 1982). If not controlled, the peachtree borer, *Synanthedon exitiosa* (Say), and the lesser peachtree borer, *S. pictipes* (Grote and Robinson) can destroy entire orchards of fruit trees. Several species in the genera *Vitacea* Engelhardt, *Melittia* Hübner and *Pennisetia* Dehne can cause serious losses to various crops (Solomon & Dix 1979).

Morphological classification of Sesiidae has undergone several revisions since the 1960s (Naumann 1971, Bradley et al. 1972, Bradley & Fletcher 1974,

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# Genetic Analysis of *Periplaneta americana* (Blattodea: Blattidae) in Central Texas Using the ITS1 Region[1]

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**ABSTRACT** American cockroaches, *Periplaneta americana* (Blattodea: Blattidae) (L.), are common pests of urban environments. Analyzing spatial distribution of *P. americana* populations in an artificial, outdoor environment provided insight of gene flow among populations collected in central Texas. This information provides a better understanding of how and if populations were segregated, or if there was a single unified population. Populations can be genetically differentiated through determining variation of specific gene regions within populations. This study revealed a ubiquitous distribution of cockroach populations, and their ability to indiscriminately inhabit areas within an urban environment. Overall, cockroaches were identified from a large interbreeding population with no discernable relationship between genetic variation of *P. americana* and spatial distribution.

**KEY WORDS** *Periplaneta americana*, ITS1 region, central Texas

Cockroaches can passively and actively disperse to new locales (Jobet et al. 2000). Gene flow may be caused by long range passive travel, such as cockroaches traveling in properties of people moving from one location to another. Active movement appears to be confined to temperate climate zones when alternative, ideal habitats are within close proximity (Cloarec et al. 1999). Schoof and Siverly (1954) indicated a lack of dispersal among American cockroach, *Periplaneta americana* (L.) (Blattodea: Blattidae), populations through sewer systems in Phoenix, Arizona, USA. This inability to disperse may have resulted from the ideal habitat a sewer system provided, including ample amounts of water, food, and harborage. It appeared that when requisites for life were fulfilled the necessity to actively disperse was reduced.

Genes usually occur in repeating, tandem units and have NTS regions between repeating segments of RNA, while ITS regions separate genes within each strand. Despite looking at the lesser of the two variable spacer regions, ITS regions still can provide an ample amount of variation to reveal a relatively moderate level of gene flow amongst the given cockroach population in central Texas (Mukha et al. 2007).

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Defining a population depends on several factors such as spatial distribution, structures from which collections were made, ecological niches occupied by a population, or the general bias of the collector(s) may contribute to the definition of a "population." Differences in allelic frequencies may also be used to distinguish populations. Hypothetically, genetic variability decreases in populations secluded from other populations (Cloarec et al. 1999). In regards to cockroaches, isolated populations may have limited gene fluctuation because of minimal migration from outside populations contributing to the non-diverse gene pool (Mukha et al. 2007).

Only a few cockroaches are needed to establish a new population in a given area. Mukha et al. (2007) identified three *Blattella germanica* (Linnæus) (Blattodea: Blattidae) populations with substantial genetic differentiation, hence, isolated populations separated between 15 and 115 km. In contrast, Cloarec et al. (1999) analyzed isoenzymatic genetic markers from *B. germanica* populations from two French cities (Rennes and Sète) approximately 900 km apart and demonstrated limited genetic variation. Consequently, due to contrasting results in previous studies it is inconclusive as to whether or not populations analyzed over distances are homologous.

Genetic variation among dispersing populations may result from various genetic events. Genetic drift, founder effects, natural selection, migration, and gene flow are some factors that might contribute to genetic variation (Jobet et al. 2000). Founder effects occur more frequently in cockroach populations because only a limited number of individuals are required to establish new populations (Cloarec et al. 1999). Cloarec et al. (1999) suggested that populations within a defined geographical area (i.e., a city) were more homologous than populations compared between greater distances (i.e., city to city). Populations separated by variable distances retaining similar allelic frequencies indicated a homologous correlation between populations, hence, gene flow (Cloareac et al. 1999).

The objective of this study was to determine gene flow among populations collected in central Texas. This information may allow for a better understanding of how and if populations were segregated, or if there was a single unified population.

## Materials and Methods

**Sampling technique for cockroaches.** *Periplaneta americana* (L.) were collected within 50 m of neighboring urban structures in College Station, TX and investigated for potential gene flow by phylogenetic analysis among the collected population(s). Collecting sites on campus were selected from locations with the highest cockroach populations based on preliminary trapping. Once locations were established, three collecting containers were placed within a 1.83 $m^2$ square at each trapping location. Coordinates of each site were determined with a Gormin eTrex® Vista Cx GPS unit (Garmin Ltd., Olathe, KS, USA). Additional samples from the following cities in Texas were obtained from the Texas A&M University Insects in Human Society (ENTO 322) Student Insect Collection including: Pleasanton, Del Rio, Bryan, and Hempstead, Texas. The cockroaches from the Texas A&M University Insects in Human Society Student Insect Collection were preserved by pinning and stored in boxes turned in by the

students. Data points for all cockroaches collected were uploaded to Google Earth.

Containers used for collection at College Station, TX were glass mason jars (430 ml) coated with Elmer's Acid Free Craft Bond (Elmer's Products, Inc., Columbus, Ohio, USA) and rolled in Quickrete® Playsand (Quickrete® International, Inc., Atlanta, GA, USA), according to Granovsky (1983). The top 2 cm of the jar opening was lined with H-E-B brand petroleum jelly (H-E-B, San Antonio, TX, USA), and baited with approximately 51.76 ml beer (Miller Brewing Co., Milwaukee, WI, USA) and 7.04 g of H-E-B brand white bread (H-E-B, San Antonio, TX, USA) for specimen collections (Barcay 2004). Baited containers were placed in the field immediately after adding the beer/bread mixture. Jars were set out prior to dusk and collected from the field after 8–12 h.

Cockroaches were collected from each jar and stored in individual plastic bags (16.5 × 14.9 cm) with up to three plastic bags containing cockroaches from each site. Collected specimens were stored in a freezer at −20°C until further analyses were conducted.

**Molecular analysis.** Molecular probes were used to identify different haplotypes within each cockroach sample. The hind femur from each specimen was used for genetic analysis. The specific region providing the greatest amount of information about the genetic flow is the ITS1 region located between the 18S and 5.8S gene. The success of fragments of both the 18S and 5.8S genes, and the entire IST1 region, to make up the probe in identification of individuals and their genetic composition from the provided specimens has been demonstrated in recent studies (Mukha et al. 2007).

A 562-bp section of the nuclear 3′ portion of 18S rDNA, all of ITS1 region, and the 5′ portion of 5.8S were amplified with the primers rDNA2 (5′-TTGATTACGTCCCTGCCCTTT-3′) and rDNA 1.58S (5′-GCCACCTAGTGAGCCGAGCA-3′) with a thermal cycler profile consisting of 40 cycles of 94°C for 45 s, 53°C for 1 min and 72°C for 1 min as described by Szalanski and Owens (2003) (Vrain et al. 1992, Cherry et al. 1997). Amplified DNA from individual cockroaches was purified and concentrated with minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega). Samples were sent to the University of Arkansas Medical School DNA Sequencing Facility (Little Rock, AR, USA) for direct sequencing in both directions. Consensus sequences were derived from both of DNA sequences from an individual with Bioedit 5.09 to verify nucleotide polymorphisms (Hall 1999).

DNA sequences were aligned by CLUSTAL W (Thompson et al. 1994). The distance matrix option of PAUP* 4.0b10 was used to calculate genetic distances according to the Kimura 2-parameter model of sequence evolution (Kimura 1980, Swofford 2001). Maximum likelihood and unweighted parsimony analysis on the alignments were conducted by PAUP* 4.0b10 (Swofford 2001). Gaps were treated as missing characters for all analysis. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1,000 resamplings with the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, the default likelihood parameters were used (HKY85 six-parameter model of nucleotide substitution, empirical base frequencies with the exception of the transition/transversion ratio, will be determined). These parameters were used to carry out a heuristic search by PAUP* with a neighbor joining tree as the starting tree. Gene flow was

**Table 1. Sample sites and haplotypes frequencies from each collection site within Texas counties.**

| City (County) | N | Haplotype (frequency) |
|---|---|---|
| Pleasanton (Atascosa) | 1 | 17(1) |
| Bryan (Brazos) | 2 | 1(1), 5(1) |
| College Station (Brazos) | 48 | 1(10), 2(1), 3(14), 4(1), 6(1), 7(1), 8(1), 9(1), 10(4), 11(1), 12(1), 13(1), 14(3), 15(1), 16(2), 18(1), 19(1), 20(1), 21(1), 22(1) |
| Hempstead (Waller) | 1 | 1(1) |

evaluated applying Mitochondrial DNA haplotypes aligned by MacClade v4 (Sinauer Associates, Sunderland, MA). Haplotype distribution between populations, number of haplotypes, number of unique haplotypes, haplotype diversity (h), and nucleotide diversity (pi) was calculated with DNAsp v3.51 and Genealogical relationships among haplotypes were constructed using TCS, with the method described by Templeton et al. (1992) (Rozas & Rozas 1999, Clement et al. 2000).

## Results

DNA sequencing of the ITS1 region from 52 sampled cockroaches resulted in an average size of 560 bp. There were 22 haplotypes observed from four Texas counties with the 3 haplotype being the most common (Table 1). There were 25 unique haplotypes. Del Rio, Texas is approximately 462 km from College Station; Pleasanton, Texas has a distance of approximately 274 km from College Station, Texas; Hempstead, Texas is approximately 62 km away from College Station, Texas; Bryan, Texas is a sister city to College Station, Texas separated by approximately 8 km.

There were 41 polymorphic sites (Table 2). The average number of pairwise nucleotide differences was 3.992. Out of the 22 haplotypes there were 25 singletons or unique sequences. Nucleotide diversity, $\pi$, was 0.007, and the mean number of pairwise nucleotide differences between haplotypes, $k$, was 3.992. Tajima's D test of neutrality of mutations against excess of recent mutations were not significant (Table 3).

Applying PAUP* version 4.0b10 software, both Neighbor-Joining (NJ) and Maximum Parsimony (MP) analyses were conducted. Results of the NJ tree using uncorrected "P" distances are presented as an unrooted cladogram (Figure 1). For MP analysis, parametric bootstrapping (50% majority-rule) with a full heuristic search was employed for 1000 pseudoreplicates with a starting seed = 632095753. A total of 560 characters were evaluated with all characters equally weighted; 513 characters remained constant and 20 characters were parsimony informative. Gaps in nucleic sequences were treated as "missing" with the starting tree(s) obtained via stepwise addition. The Branch-swapping algorithm: tree-bisection-reconnection (TBR) was employed. The sum of minimum possible

lengths = 48; the sum of maximum possible lengths = 140. A single tree (Figure 1) was produced with length = 113, CI = 0.425 and RI = 0.293. Uncorrected ("P") distances were used to construct the NJ tree.

Phylogenetic trees were also obtained using a Bayesian analysis with the GTR+G model by applying Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.4.7 software (Drummond & Rambaut 2007). For Bayesian inference, four Markov chains run for $10^6$ generations with a burn-in of $2 \times 10^4$ were used to reconstruct the consensus tree (Figure 2); MP branch support are presented above the major branches with posterior bootstrapping probabilities presented behind each node (Figure 2).

TCS spanning tree analysis reveled that haplotype 3 had the highest outgroup possibility for all of the 22 haplotypes (Figure 3)

## Discussion

The purpose of this study was to analyze the spatial distribution of *P. americana* populations in an outdoor, urban environment and to determine the extent of gene flow among the populations. This study attempted to determine genetic variability among *P. americana* collected on Texas A&M University in College Station, TX.

Genetic differentiation occurs between populations in diverse locations for all organisms (Austin et al. 2004). Inward et al. (2007) suggested that the orders Isoptera and Blattodea are related, thus their genes would coalesce to a single common ancestor. It can be assumed that the individual lineages would comprise similar genetic material, thus specific gene regions would be applicable for amplification purposes in both orders. Phylogenetic studies and population genetics performed on termites commonly used the 16S region of the gene for amplification. The 16S region of the gene was initially chosen as the amplification site in this study to determine variability among cockroach populations collected on campus. During this study, the 16S gene region amplification protocol commonly used in termite studies failed to amplify cockroach DNA. Differing genetic compositions of the 16S gene region selected may have resulted from evolution of separate ordinal lineages over time. The universal primers that annealed for termite DNA simply would not work for cockroach DNA and/or the annealing temperature may have been to low thus inhibiting annealing or too high which would damage the primers or DNA. Consequently, the ITS1 region was chosen for amplification because of the availability of comparable sequences available on Genbank (National Center for Biotechnology Information).

The ITS1 region functions in primary rRNA processing and has a higher rate differentiation than the 18S gene region of rRNA (James et al. 1996). Mukha et al. (2007) reported rRNA genes as being the most conserved among populations, while non-transcribed spacer regions have the most variation, and transcribed spacer regions between the two extremes. There are conflicting results when analyzing the ITS1 region for genetic variability in insect populations. Szalanzski et al. (2008) determined a lack of diversity in the nuclear gene region (ITS1 region) with high levels of differentiation when examining the mitochondrial DNA region (16S gene) in *Cimex lectularius* (L.) (Hemiptera: Cimicidae). The ITS1 region may have indicated low levels of diversity in this species at these specific loci (Szalanzski et al. 2008). When the ITS1 region was used, it failed to

**Table 2. Base pair differences between *P. americana* haplotypes from Texas.**

| | | Nucleotide site | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | N | 27 | 33 | 52 | 55 | 58 | 67 | 69 | 82 | 85 | 92 | 136 | 137 | 179 | 186 | 198 |
| 1 | 12 | T | T | C | C | A | C | A | C | C | A | C | G | C | C | T |
| 2 | 1 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 3 | 14 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 4 | 1 | * | * | * | * | * | G | * | * | * | * | * | * | * | * | * |
| 5 | 1 | * | * | * | * | G | G | G | * | * | * | * | * | * | * | C |
| 6 | 1 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 7 | 1 | * | * | * | * | * | * | * | * | * | C | * | * | A | * | * |
| 8 | 1 | * | C | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 9 | 1 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 10 | 4 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 11 | 1 | C | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 12 | 1 | * | * | T | T | * | G | * | T | T | C | T | * | A | T | * |
| 13 | 1 | * | * | T | * | * | * | * | T | T | * | T | * | * | * | * |
| 14 | 3 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 15 | 1 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 16 | 2 | * | * | * | * | * | G | * | T | * | * | * | * | * | * | * |
| 17 | 1 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 18 | 1 | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 19 | 1 | * | * | * | * | * | G | G | * | * | * | T | T | * | * | * |
| 20 | 1 | * | * | T | T | * | * | * | T | T | * | T | * | A | T | * |
| 21 | 1 | * | * | T | T | * | * | * | T | T | * | T | * | A | T | * |
| 22 | 1 | * | * | T | * | * | * | * | T | T | C | T | * | * | * | * |

| | | Nucleotide site | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | N | 199 | 225 | 239 | 264 | 272 | 303 | 314 | 355 | 366 | 437 | 463 | 488 | 514 | 515 |
| 1 | 12 | T | A | G | G | G | G | A | A | G | C | A | C | A | A |
| 2 | 1 | * | * | * | * | * | * | T | T | * | T | * | * | C | * |
| 3 | 14 | * | T | * | * | * | * | * | * | * | * | * | * | * | * |
| 4 | 1 | * | T | * | * | * | * | * | * | * | * | * | * | * | * |
| 5 | 1 | * | T | * | * | * | * | * | * | * | * | * | * | * | * |
| 6 | 1 | * | T | * | * | * | * | * | * | * | * | * | * | * | T |
| 7 | 1 | * | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 8 | 1 | * | * | * | C | * | * | * | * | * | * | * | * | * | * |
| 9 | 1 | A | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 10 | 4 | A | T | * | * | * | * | * | * | * | * | * | * | * | * |
| 11 | 1 | * | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 12 | 1 | A | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 13 | 1 | A | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 14 | 3 | * | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 15 | 1 | A | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 16 | 2 | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| 17 | 1 | * | * | G | * | G | C | * | * | C | * | C | * | * | * |

**Table 2. Continued.**

| Haplotype | N | Nucleotide site | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 199 | 225 | 239 | 264 | 272 | 303 | 314 | 355 | 366 | 437 | 463 | 488 | 514 | 515 |
| 18 | 1 | * | * | * | * | * | * | * | * | C | * | * | G | * | * |
| 19 | 1 | * | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 20 | 1 | * | * | G | * | * | * | * | * | * | * | * | * | * | T |
| 21 | 1 | * | * | G | * | * | * | * | * | * | * | * | * | * | * |
| 22 | 1 | * | * | G | * | * | * | * | * | * | * | * | * | * | * |

determine phylogenetic relationships between *Reticulitermes* termites (Tripodi et al. 2006). On the other hand, there was sufficient variability in the ITS1 region used to identify diversity among *Diabrotica* (Coleoptera: Chrysomelidae) species (Szalanski & Owens 2003). Additionally, Szalanski et al. (2000) demonstrated differentiation between *Nicrophorus americanus* (Olivier) (Coleoptera: Silphidae) based on results from the ITS1 region. The current study may have demonstrated biotic homogenization within populations of *P. americana* based on data from the ITS1 region (McKinney & Lockwood 1999).

Haplotypes are defined by at least a single nucleotide difference within the same gene region between sequences thus identifying unique genes. Haplotype diversity is the number of haplotypes compared to their relative frequency and determined the probability of two sequences chosen from a population being different (Austin et al. 2004). Tajima's D is a statistical determination of the neutral mutation hypothesis in natural populations (Tajima 1989). Positive values of D indicate population bottlenecks while negative values of D suggest expansion of a population (Tajima 1989). Nucleotide diversity (Pi) in populations assumed neutrality based on the infinite alleles model (Austin et al. 2004).

Among the 52 cockroaches sampled there were 22 haplotypes indicating a high amount of variation in the population. TCS spanning tree analysis defined lineages from nuclear markers which implied populations moderate levels of gene flow. The lack of isolated populations was reconfirmed by maximum likelihood and Baysian phylogenetic analyses.

*Periplaneta americana* samples from Bryan, College Station, Hempstead, and Pleasanton, TX were in a single clade, including *P. americana* sequence obtained from Genbank (AF321248). Sequence comparisons reconfirmed speciation and

**Table 3. Summary of statistics for rDNA genetic variation.**[a]

| Sample | $n$ | $h$ | $s$ | Hd | $\pi$ ($k$) | $\theta_s$ | $\theta_g$ | $D^{+}$** | $F^{+}$** | D* |
|---|---|---|---|---|---|---|---|---|---|---|
| Texas | 52 | 28 | 41 | 0.918 ± 0.025 | 0.007 (3.992) | 0.017 | 9.29 | −3.39 | −3.41 | −1.94 |

*P < 0.05; **P < 0.02.

[a]$n$ is the number of sequences, $h$ is the number of haplotypes, $s$ is then number of polymorphic sites, Hd is haplotype diversity ± SD, $\pi$ is nucleotide diversity, $k$ is mean number of pairwise nucleotide differences, $\theta_s$ is the theta per site, $\theta_g$ is theta per gene, $D^{+}$ and $F^{+}$ are statistics per Fu and Li, and $F^{+}$ is Tajima D statistic.

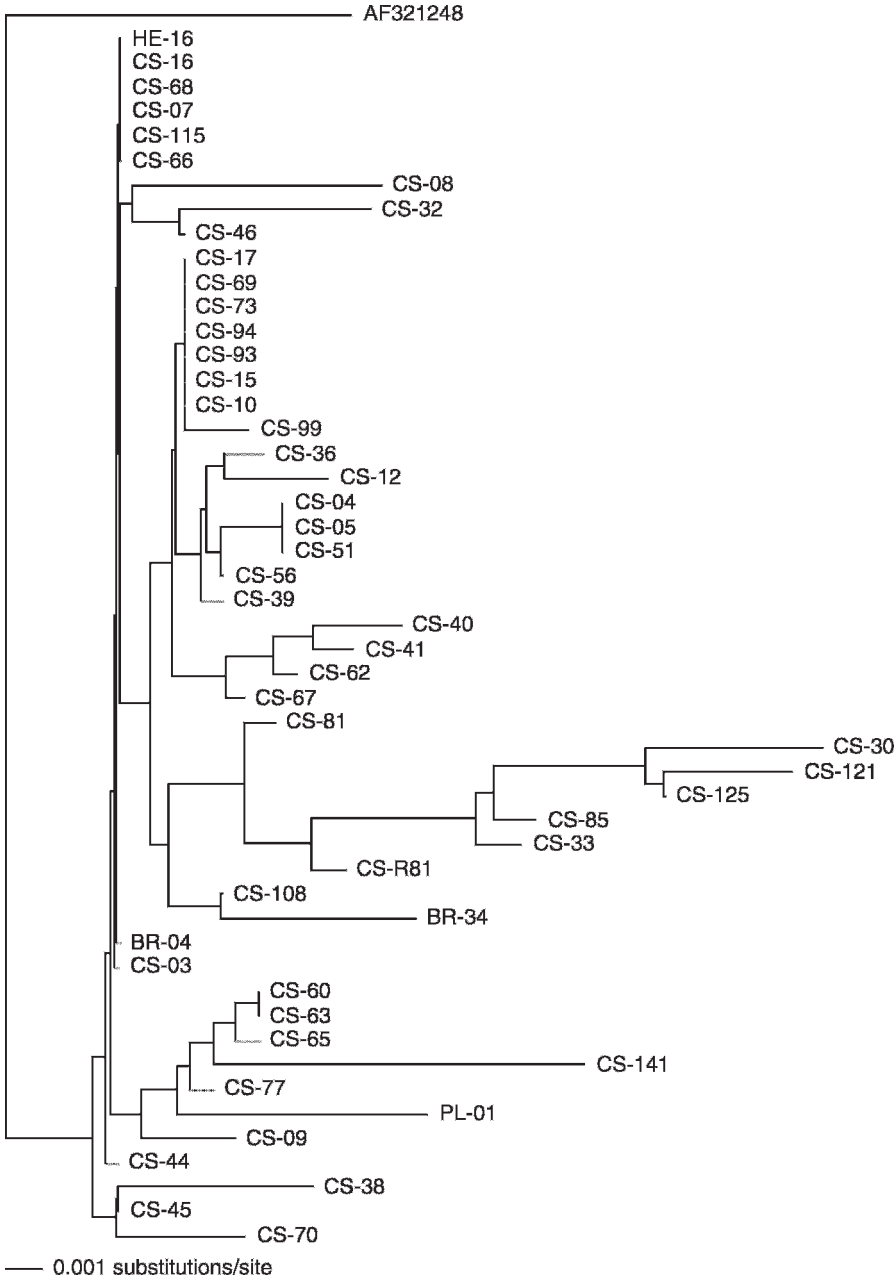


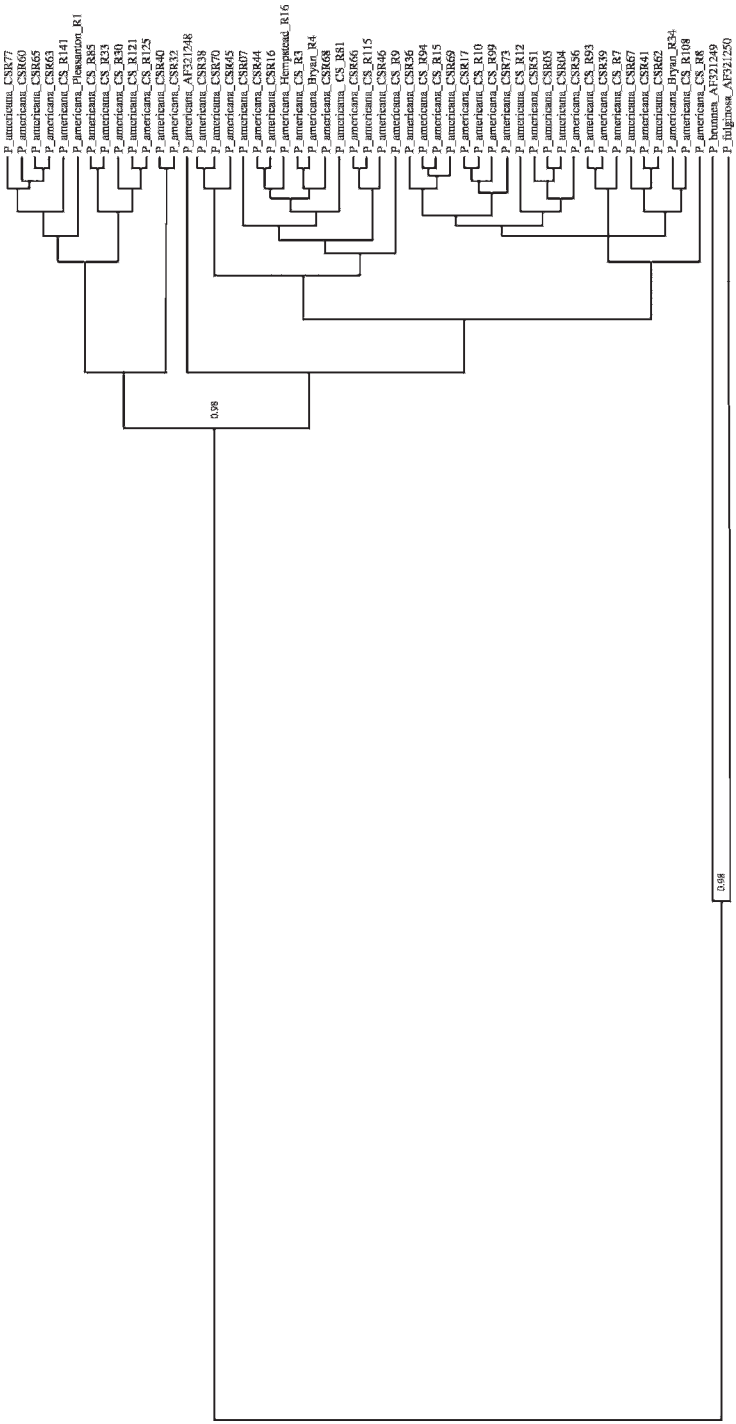

**Fig. 1.** Phylogenetic relationship of *P. americana* rDNA ITS1 region. Neighbor-joining tree with a length = 113, CI = 0.425, and RI = 0.293 resulting from samples collected from quadrants on the Texas A&M University campus College Station, Texas, and from Bryan, Hempstead, and Pleasanton, Texas.

revealed moderate interbreeding between *P. americana*. The Smokey Brown cockroach (*Periplaneta fuliginosa*) (Serville) and Brown cockroach (*Periplaneta brunnea*) (Burmeister) were chosen as outliers because their sequences were available on Genbank, AF321250 and AF321249, respectively, and are members of the same genera as are American cockroaches. Comparing various species allowed a broader analysis of *P. americana* to varying genetic sequences as a result of speciation within the same genera. Comparing the 52 sequences amplified to 22 haplotypes suggested a moderate amount of variation in the population based on nuclear markers. The lack of isolation indicated interbreeding populations on campus. Differentiation of genetic variation based on spatial distribution of *P. americana* populations indicated the success and ability of breeding with independence among various populations.

Migration of individuals to new locations provided opportunities for new genetic material to be introduced into a population thus increasing some haplotypic diversity. Szalanski and Owens (2003) suggested lack of variation among southern corn rootworm resulted from motility or population expansion. Diversity among populations collected on campus most likely resulted from the ability of cockroaches to travel successfully in urban environments and breed effectively with cockroaches from other areas thus contributing to a constant influx of genetic material into various populations. It remains unknown what degree of genetic variability is observed among other cockroach species.

Genetic variability in populations can be achieved through genetic drift, genetic flow, natural selection, and founder effects (Slatkin 1987). Genetic drift can affect nuclear genes though the fixation of loci in various locations, but gene flow can impede the permanent fixation of the alleles (Slatkin 1987). Lenormand (2002) determined gene flow limited adaptation of genes to specific locations because new genes from outside sources prevent loci from becoming fixed in the environment. Gene flow can prevent speciation because introduced genetic material can be adapted for survival in a particular environment differing from the population in which it emigrated (Slatkin 1987). Gene flow is an indirect method of determining movement within a population. Bossart and Prowell (1998) indicated that the only method that definitively determined gene flow among a population was through the use of genetic tags used to track movement which had been successful in marine organisms. Cloarec et al. (1999) described gene flow (in *B. germanica*) from the movement of cockroaches over long distances by passive transportation, thus increasing the rate of homogenization among the genetic material between populations. Results found in the current study were similar to those found in Cloarec et al. (1999) when they determined that the German cockroach populations were not isolated in two French cities 900 km apart. Mukha et al. (2007) determined three *B. germanica* populations found in farms separated by 10–100 km and had three populations differentiated by rDNA markers, but they were still not completely isolated. Species, including highly mobile organisms such as cockroaches, disperse through an environment until geographical impediments such as oceans, deserts, and mountains limit expansion (Slatkin 1987).

To date, this study is the first using rDNA markers to identify spatial relationships and gene flow among *P. americana* populations in the United States. Future studies may analyze a broader range of genes including mitochondrial DNA to determine if there are lineages formed by maternal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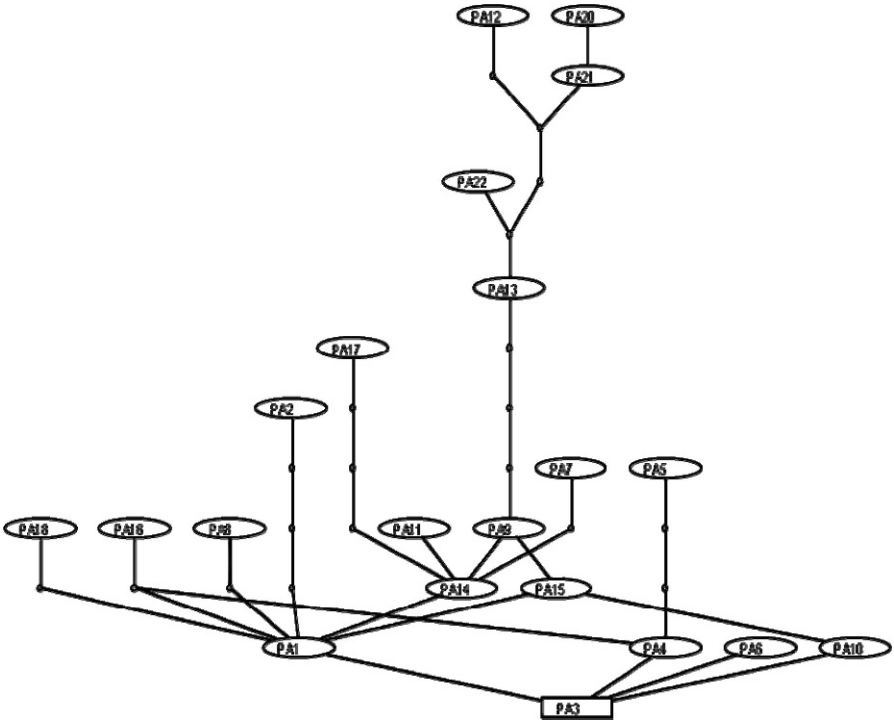


**Fig. 3.** Genealogical relationship among haplotypes of *P. americana* estimated by TCS. The square is the most baysesian haplotype among the collected populations in Texas. Ovals are haplotypes not observed and each branch represents a single mutation.

genetic material. Also, analyzing gene flow at several differing sequences within DNA may determine a more comprehensive evolutionary lineage of divergences in cockroach populations.

## Acknowledgments

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**Fig. 2.** Phylogenetic trees using a Bayesian analysis with MP branch support are presented above the major branches with posterior bootstrapping probabilities presented behind each node for samples collected from quadrants on the Texas A&M University campus College Station, Texas, and from Bryan, Hempstead, and Pleasanton, Texas.

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# Genetic Analysis of *Periplaneta americana* (Blattodea: Blattidae) in Central Texas Using the ITS1 Region[1]

Jennifer L. Pechal,[2,3] James W. Austin,[5] Allen L. Szalanski,[4] Roger E. Gold,[3] and Jeffery K. Tomberlin[3]

J. Agric. Urban Entomol. 25(3): 179–191 (July 2008)

**ABSTRACT** American cockroaches, *Periplaneta americana* (Blattodea: Blattidae) (L.), are common pests of urban environments. Analyzing spatial distribution of *P. americana* populations in an artificial, outdoor environment provided insight of gene flow among populations collected in central Texas. This information provides a better understanding of how and if populations were segregated, or if there was a single unified population. Populations can be genetically differentiated through determining variation of specific gene regions within populations. This study revealed a ubiquitous distribution of cockroach populations, and their ability to indiscriminately inhabit areas within an urban environment. Overall, cockroaches were identified from a large interbreeding population with no discernable relationship between genetic variation of *P. americana* and spatial distribution.

**KEY WORDS** *Periplaneta americana*, ITS1 region, central Texas

Cockroaches can passively and actively disperse to new locales (Jobet et al. 2000). Gene flow may be caused by long range passive travel, such as cockroaches traveling in properties of people moving from one location to another. Active movement appears to be confined to temperate climate zones when alternative, ideal habitats are within close proximity (Cloarec et al. 1999). Schoof and Siverly (1954) indicated a lack of dispersal among American cockroach, *Periplaneta americana* (L.) (Blattodea: Blattidae), populations through sewer systems in Phoenix, Arizona, USA. This inability to disperse may have resulted from the ideal habitat a sewer system provided, including ample amounts of water, food, and harborage. It appeared that when requisites for life were fulfilled the necessity to actively disperse was reduced.

Genes usually occur in repeating, tandem units and have NTS regions between repeating segments of RNA, while ITS regions separate genes within each strand. Despite looking at the lesser of the two variable spacer regions, ITS regions still can provide an ample amount of variation to reveal a relatively moderate level of gene flow amongst the given cockroach population in central Texas (Mukha et al. 2007).

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# *In vitro* Inhibition of Carboxylesterases by Insecticides and Allelochemicals in *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae) and *Clostera anastomosis* (L.) (Lepidoptera: Notodontidae)[1]

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**ABSTRACT** Both *Micromelalopha troglodyta* (Graeser) and *Clostera anastomosis* (L.), are important pests of poplar, and usually coexist during the period of poplar growth in China. The carboxylesterases have been recognized to play an important role in the detoxification of xenobiotics in the two Notodontidae species. *In vitro* inhibitory effects by insecticides and allelochemicals on carboxylesterase activity in *M. troglodyta* and *C. anastomosis* were studied. The results showed that three organophosphates (chlorpyrifos, phoxim and profenofos) were the best inhibitors of the enzymes among all compounds tested in *M. troglodyta* and *C. anastomosis*. Chlorpyrifos inhibited carboxylesterase activity in *M. troglodyta* and *C. anastomosis* to a similar degree, but phoxim and profenofos inhibited to different degrees. Furthermore, kinetic analyses of carboxylesterase inhibition by phoxim, chlorpyrifos and profenofos were also investigated. The results showed that phoxim inhibited carboxylesterase activity with respect to α-naphthyl acetate (α-NA) in a noncompetitive manner in *M. troglodyta*, while the inhibition of phoxim showed competitive to α-NA in *C. anastomosis*. The inhibition of chlorpyrifos showed neither typical competitive nor noncompetitive to α-NA both in *M. troglodyta* and *C. anastomosis*. Profenofos inhibited carboxylesterase activity with respect to α-NA in a noncompetitive manner in *M. troglodyta*, while the inhibition of phoxim showed neither typical competitive nor noncompetitive to α-NA in *C. anastomosis*. These results may contribute to the understanding of the sensitivity difference of *M. troglodyta* and *C. anastomosis* to pesticides, and could provide the basis for integrated pest management of the two Notodontidae species.

**KEY WORDS** *Micromelalopha troglodyta* (Graeser), *Clostera anastomosis* (L.), Lepidoptera, Notodontidae, carboxylesterases, allelochemicals, insecticides

Both *Micromelalopha troglodyta* (Graeser) and *Clostera anastomosis* (L.) are important pests of poplar, causing damage by direct feeding, and usually coexist during the period of poplar growth in China. The control of *M. troglodyta* and *C. anastomosis* is primarily dependent on the application of insecticides. Difference in the efficacy of insecticides between the two Notodontidae species has been

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observed. Carboxylesterases (EC 3.1.1.1), an ubiquitous B-esterase (Barron et al. 1999), have been recognized to play an important role in the difference in efficacy of insecticides.

Carboxylesterases catalyze xenobiotic compounds into their corresponding free acid, and are thus involved in the detoxication and metabolism of xenobiotic compounds (Ketterman et al. 1989, Saboori & Newcombe 1990, Teng & Sun 2003, Gao et al. 2007) and insecticides (Chambers & Chambers 1990, Chanda et al. 1997, Clement 1984, Jokanocic 1989). The expression of carboxylesterases is highly correlated with changes in insecticide sensitivity (Whyard et al. 1994, 1995, Benke & Murphy 1975, Karanth & Pope 2000, Moser et al. 1998). Zhang et al. (2007) found that carboxylesterases play an important role for the pyrethroid resistance in house fly, *Musca domestica* (L.). Therefore, the inhibition of carboxylesterases can be important in toxicity with exposure to insecticides (Cohen 1984, Gupta & Dettbarn 1993, Karanth et al. 2001).

Very little is known about the biochemical properties of carboxylesterases in *M. troglodyta* and *C. anastomosis*. In the present work, the results of some of the biochemical difference of this enzyme in these two kinds of pests are reported for the first time. These results may contribute to the understanding of the sensitivity difference of *M. troglodyta* and *C. anastomosis* to pesticides and help determine their role in the metabolism and excretion of pesticides. Another purpose of this study is to find some potential carboxylesterase inhibitors which might be used as synergists to enhance the toxicity of insecticides.

## Materials and Methods

**Chemicals.** α-naphthyl acetate (α-NA), α-naphthol, 2-tridecanone, Fast Blue BB, eserine and tannic acid (>99%) were purchased from Sigma (St. Louis, MO), Quercetin (99%) from Shanghai chemical stock (Shanghai, China), triazophos (92.0%) from Jiangxi Kaifeng Chemical Co., Ltd (Jiangxi, China), malathion (95.0%) from Hebei Shiji Pesticide Co., Ltd (Hebei, China), chlorpyrifos (97.0%) from Dow AgroSciences LLC (Indiana, USA), phoxim (99.0%) and profenofos (90%) from Tianjin Pesticide Co., Ltd (Tianjin, China), omethoate (92.0%) from Hangzhou Qingfeng Agrochemicals Co., Ltd (Zhejiang, China), methomyl (98.0%) and imidacloprid (95.0%) from Hubei Sanongda Co., Ltd (Hubei, China), fenpropathrin (92.0%) and beta-cypermethrin (97.0%) from Shandong Dacheng Pesticide Co., Ltd (Shandong, China), bifenthrin (97.0%), deltamethrin (99.0%) and cyfluthrin (92.0%) from Jiangsu Yangnong Chemical Group Co., Ltd (Jiangsu, China), lambda-cyhalothrin (95.0%) from Jiangsu Huangma Pesticide & Chemical Co., Ltd (Jiangsu, China), endosulfan (90%) from Jiangsu Rudong Pesticide Co., Ltd (Jiangsu, China), hexaflumuron (95.0%) from East Romble Agrochem (Shan Dong) Co., Ltd (Shandong, China), emamectin benzoate (90.0%) from Zhejiang Qianjiang Biochemical Co., Ltd (Zhejiang, China), abamectin (95.3%) from Shandong Jingbo Agrochemicals Co., Ltd (Shandong, China), fipronil (90.0%) from Anhui Huaxing Chemical Industry Co., Ltd (Anhui, China), acetamiprid (96.0%) from Qingdao Haili'er Medicine Co., Ltd (Shandong, China), and pyridaben (95.0%) from Shandong Sino-Agri United Biotechnology Co., Ltd (Shandong, China). Allelochemicals and insecticides were dissolved in absolute acetone. All other chemicals and solvents used were of analytical grade and purchased from commercial sources.

**Insects.** *Micromelalopha troglodyta* and *C. anastomosis* populations were collected from Kunshan, Jiangsu Province, China. They were reared in the conditioned room maintained at 26 ± 1°C, 75% RH and a 12:12 LD photoperiod. The newly molted 5th-instar larvae were used in all studies.

**Determination of carboxylesterase activity.** *Micromelalopha troglodyta* and *C. anastomosis* were homogenized in ice-cold phosphate buffer (0.04 M, pH 7.0), respectively. The homogenates were centrifuged at 4°C, 12,000 × *g* for 15 min. Then the supernatants were used for subsequent esterase activity assay. Carboxylesterase activities were assayed with α-naphthyl acetate (α-NA) as substrate according to van Aspern (1962). The working concentration of the substrates is 0.3 mM (containing 0.3 mM eserine), diluted from their respective 0.03 M stock. The assay mixture for the substrates contains 200 μL of enzyme preparation, 100 μL of 0.04 M phosphate buffer, and 1.20 mL of 0.3 mM substrate solution. The reaction was stopped by the addition of 350 μL of stop solution (two parts of 1% Fast Blue BB and five parts of 5% sodium dodecyl sulfate) after incubating at 30°C for 15 min. The color was allowed to develop for 15 min at room temperature, and the absorbance was measured at 600 nm for the hydrolysis of α-NA. Mean levels of carboxylesterase activity were based on protein content and α-naphthol standard curves. Protein content was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

**Inhibition of insecticides and allelochemicals on carboxylesterase.** All the inhibitors were dissolved in acetone in $1 \times 10^{-2}$ M and then diluted into a series of concentration with phosphate buffer (pH 7.0, 0.04 mM). Activity of carboxylesterases of *M. troglodyta* and *C. anastomosis* was determined in the present of inhibitors. The mixture of inhibitors with enzyme was incubated for 10 min at 30°C, and then followed steps of carboxylesterases. The mixture contained the correponding concentration of acetone was used as control. The $I_{50}$ values, concentrations of inhibitors required to reduce the reaction rate by 50%, were determined by linear regression of the average percent activity on the log of the inhibitor concentration (Neal & Berenbaum 1989). The experiment was replicated three times for each inhibitor.

**Kinetics of carboxylesterase inhibition by insecticides and allelochemicals.** Kinetics of carboxylesterase inhibition by chlorpyrifos, phoxim and profenofos were determined in assays containing various concentrations of α-NA and a fixed concentration of each inhibitor. The results were presented on double-reciprocal Lineweaver-Burk plots. The inhibitory constant, *K*i, the equilibrium constant for dissociation of the enzyme-inhibitor complex, was determined from plots of reciprocal velocity versus reciprocal concentration (Lineweaver-Burk plots) (Dixon & Webb 1979).

**Statistical analysis.** The results were analyzed by analysis of variance (ANOVA) with a level of significance at $P < 0.05$.

## Results

The inhibition of carboxylesterase activity by insecticides and allelochemicals is shown in Table 1. For *M. troglodyta*, all tested inhibitiors inhibited carboxylesterase activity except cyfluthrin and endosulfan. Three organophosphates (chlorpyrifos, phoxim and profenofos) were the most potent inhibitors tested, inhibiting more than 80% of carboxylesterase activity at a final concentration of 3.3 mM. Among the

**Table 1. Inhibition of carboxylesterases by insecticides and allelochemicals in *M. troglodyta* and *C. anastomosis* larvae.**

| Inhibitor (3.3 mM) | % Inhibition (means ± SD) | |
|---|---|---|
| | *M. troglodyta* | *C. anastomosis* |
| Organophosphate | | |
| Triazophos | 58.84 ± 3.24 bc A | 68.04 ± 7.16 bc A |
| Malathion | 30.38 ± 7.51 ef A | 21.49 ± 4.11 gh A |
| Chlorpyrifos | 92.45 ± 6.25 a A | 74.09 ± 2.53 ab B |
| Phoxim | 82.24 ± 0.56 a A | 84.86 ± 1.91 a A |
| Omethoate | 43.35 ± 4.05 cde A | 13.97 ± 2.83 hi B |
| Profenofos | 82.04 ± 5.90 a A | 74.65 ± 0.91 ab A |
| Carbamate | | |
| Methomyl | 21.70 ± 4.18 fg B | 56.10 ± 6.25 cd A |
| Pyrethriod | | |
| Fenpropathrin | 43.79 ± 6.14 cde A | 46.85 ± 2.56 de A |
| Beta-cypermethrin | 12.62 ± 2.73 gh A | −5.21 ± 1.96 k B |
| Bifenthrin | 55.33 ± 11.30 bcd A | 14.41 ± 2.06 hi B |
| Lambda-cyhalothrin | 3.35 ± 0.28 h B | 52.21 ± 5.82 de A |
| Deltamethrin | 11.97 ± 2.86 gh A | −12.77 ± 1.35 kl B |
| Cyfluthrin | −23.57 ± 2.15 i B | 14.91 ± 2.90 hi A |
| Organochlorine | | |
| Endosulfan | −22.94 ± 0.36 i B | 29.37 ± 3.90 fg A |
| Other insecticides | | |
| Hexaflumuron | 22.87 ± 2.94 fg A | 31.44 ± 6.92 fg A |
| Emamectin benzoate | 14.62 ± 2.90 gh A | −9.98 ± 1.41 kl B |
| Abamectin | 61.88 ± 10.32 b A | 49.82 ± 7.49 de A |
| Fipronil | 41.71 ± 0.91 de B | 75.04 ± 8.00 ab A |
| Imidacloprid | 11.93 ± 0.70 gh A | −0.45 ± 0.26 jk B |
| Acetamiprid | 47.99 ± 9.06 bcd A | 37.46 ± 2.94 ef A |
| Pyridaben | 17.57 ± 3.75 fgh A | −18.63 ± 0.75 l B |
| Allelochemical | | |
| Quercetin | 7.40 ± 0.14 gh A | 8.45 ± 1.70 ij A |
| Tannic acid | 47.42 ± 0.13 bcd A | −37.47 ± 1.66 m B |
| 2-tridecanone | 19.46 ± 3.96 fg B | 40.81 ± 3.84 ef A |

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $P < 0.05$.
Means within a column followed by the same small letter are not significantly different.
Means within a row followed by the same capital letter are not significantly different.

inhibitors tested, 3 organophosphates (triazophos, malathion and omethoate), 2 pyrethriods (fenpropathrin and bifenthrin), 1 allelochemical (tannic acid) and 3 other insecticides (emamectin, fipronil and acetamiprid) were moderately inhibitory, while 1 carbamate (methomyl), 3 pyrethriods (beta-cypermethrin, lambda-cyhalothrin and deltamethrin), 2 allelochemicals (quercetin and 2-tridecanone) and 4 other insecticides (hexaflumuron, emamectin benzoate, imidacloprid and pyridaben) were the least inhibitory.

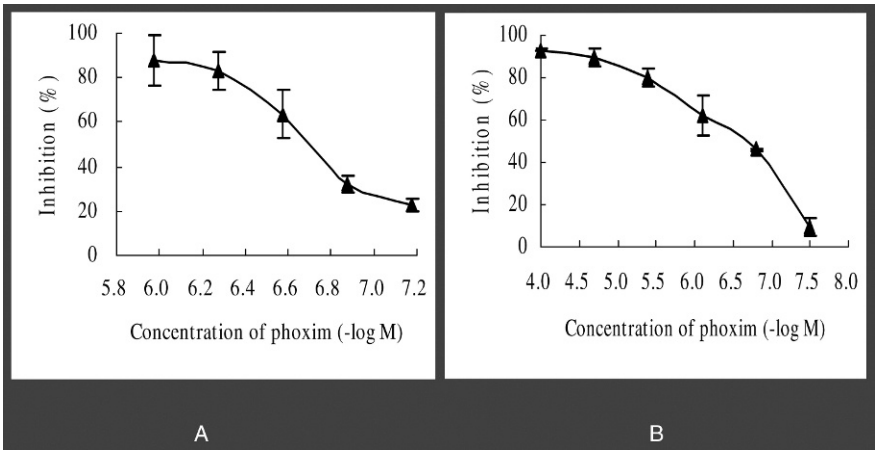


**Fig. 1.** *In vitro* inhibition of carboxylesterases by phoxim in *M. troglodyta* and *C. anastomosis* larvae. (A) *M. troglodyta*. (B) *C. anastomosis*.

For *C. anastomosis*, all insecticides inhibited carboxylesterase activity except for 6 compounds (beta-cypermethrin, deltamethrin, emamectin benzoate, imidacloprid, pyridaben and tannic acid). Four inhibitors (phoxim, fipronil, chlorpyrifos and profenofos) were the most potent inhibitors tested, inhibiting more than 70% of carboxylesterase activity at a final concentration of 3.3 mM. Among the inhibitors tested, 1 organophosphate (triazophos), 1 carbamate (methomyl), 2 pyrethriods (fenpropathrin and lambda-cyhalothrin), 1 allelochemical (2-tridecanone) and 3 other insecticides (hexaflumuron, abamectin and acetamiprid) were moderately inhibitory, while 2 organophosphates (malathion and omethoate), 2 pyrethriods (bifenthrin and cyfluthrin), 1 organochlorine (endosulfan) and 1 allelochemical (quercetin) were the least inhibitory.

The sensitivities of carboxylesterases of *M. troglodyta* and *C. anastomosis* to three insecticides (phoxim, chlorpyrifos and profenofos) were investigated and are expressed as $I_{50}$ values. Phoxim, chlorpyrifos and profenofos inhibited carboxylesterase activity *in vitro* in a dose-dependent manner in *M. troglodyta* and *C. anastomosis* (Figs. 1–3). The $I_{50}$ values of these three insecicides for carboxylesterase activity in *M. troglodyta* and *C. anastomosis* are summarized in Table 2, with $I_{50}$ values ranging from $2.07 \times 10^{-7}$ to $84.39 \times 10^{-7}$ M. Phoxim being the most potent inhibitor. Chlorpyrifos inhibited carboxylesterase activity in *M. troglodyta* and *C. anastomosis* to a similar degree, but phoxim and profenofos inhibited to different degrees.

The results of kinetic analyses of carboxylesterases inhibition by phoxim, chlorpyrifos and profenofos using Lineweaver-Burk plots are summarized in Figs. 4–6. Phoxim inhibited carboxylesterase activity with respect to α-NA in a noncompetitive manner (*V*max decreased, while *K*m remained unchanged) in *M. troglodyta*, with *K*i of $1.16 \times 10^{-3}$ mM, while the inhibition of phoxim showed competitive to α-NA (*K*m increased while *V*max remained unchanged) in *C. anastomosis*, with *K*i of $7.13 \times 10^{-4}$ mM (Fig. 4). The inhibition of chlorpyrifos showed neither typical competitive nor noncompetitive to α-NA both in *M. troglodyta* and *C. anastomosis*, with *K*i of $1.00 \times 10^{-2}$ and $8.46 \times 10^{-3}$ mM,

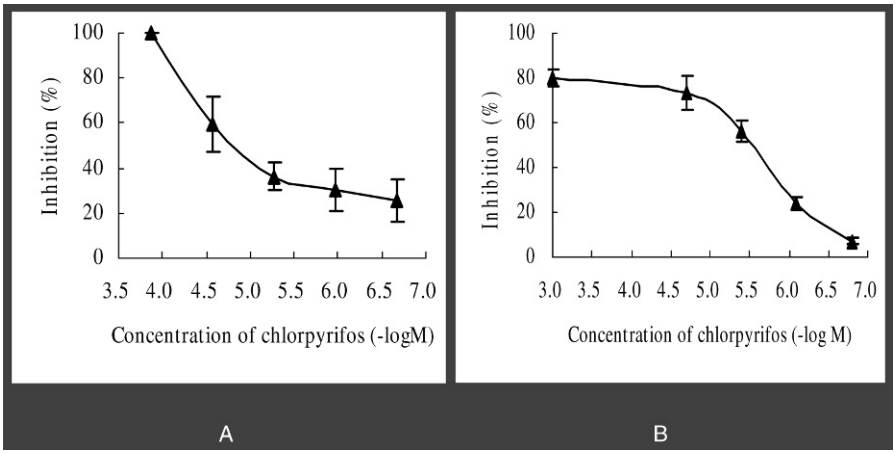


**Fig. 2.** *In vitro* inhibition of carboxylesterases by chlorpyrifos in *M. troglodyta* and *C. anastomosis* larvae. (A) *M. troglodyta*. (B) *C. anastomosis*.

respectively (Fig. 5). Furthermore, profenofos inhibited carboxylesterase activity with respect to α-NA in a noncompetitive manner (*V*max decreased, while *K*m remained unchanged) in *M. troglodyta*, with *K*i of $2.67 \times 10^{-4}$ mM, while the inhibition of profenofos showed neither typical competitive nor noncompetitive to α-NA in *C. anastomosis*, with *K*i of $9.90 \times 10^{-3}$ mM (Fig. 6).

## Discussion

Carboxylesterases are important in the detoxification of some compounds containing carboxylic esters and serves as alternative targets for anticholines-

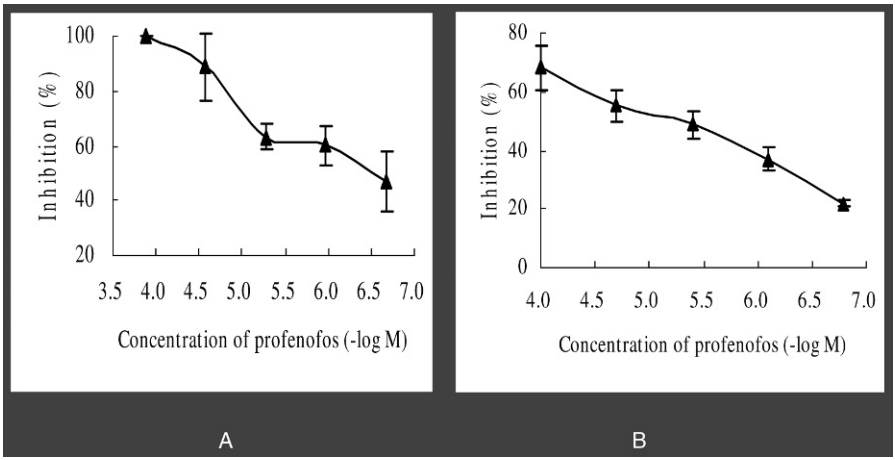


**Fig. 3.** *In vitro* inhibition of carboxylesterases by profenofos in *M. troglodyta* and *C. anastomosis* larvae. (A) *M. troglodyta*. (B) *C. anastomosis*.

**Table 2.** ***$I_{50}$* values of insecticides for carboxylesterase activity in *M. troglodyta* and *C. anastomosis*.**

| Insecticide | $I_{50}$ (means ± SD)($10^{-7}$ M) | |
|---|---|---|
| | *M. troglodyta* | *C. anastomosis* |
| Phoxim | 2.07 ± 0.30 b B | 5.16 ± 1.01 b A |
| Chlorpyrifos | 64.85 ± 4.31 a A | 84.39 ± 11.85 a A |
| Profenofos | 6.27 ± 3.45 b B | 57.60 ± 15.56 a A |

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $P < 0.05$.
Means within a column followed by the same small letter are not significantly different.
Means within a row followed by the same capital letter are not significantly different.

terase insecticides. Numerous studies have shown that the increased carboxylesterase metabolic detoxifications were involved in resistance in many insect pests. In *Bombyx mori* L., the elevated carboxylesterase activities resulted from the increase in the expression level of carboxylesterase were found to play an important role in the resistance of *B. mori* to densonucleosis virus (Gao et al. 2007). In *Nilaparvata lugens* (Stål), increased carboxylesterase activity was also found to be a major resistance mechanism against organophosphorus insecticide (Small & Hemingway 2000). Thus, controlling the activity of these enzymes would be highly useful for management of the biological impact of ester compounds consumed by insects through different ways. Therefore, *in vitro* inhibition of carboxylesterases by insecticides and allelochemicals in *M. troglodyta* and *C. anastomosis* larvae was studied. These results may contribute to the understanding of the sensitivity difference of these two pests to pesticides, and could provide the basis for integrated pest management of the Notodontidae species.

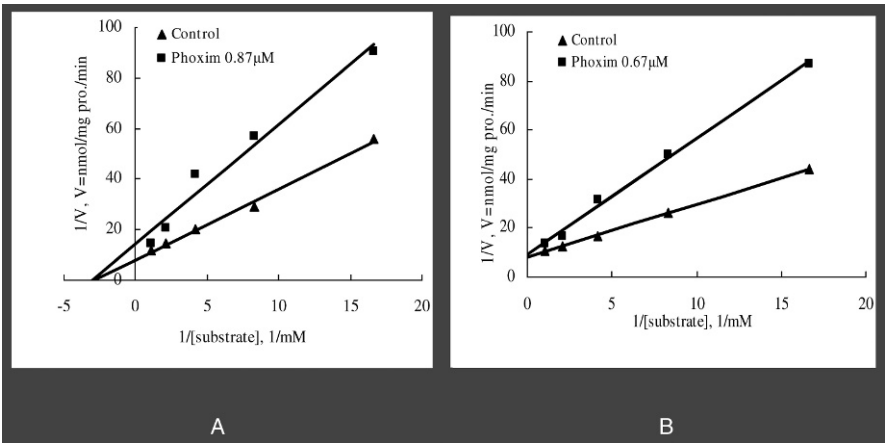


**Fig. 4.** Kinetics of carboxylesterases inhibition by phoxim in *M. troglodyta* and *C. anastomosis* larvae. (A) *M. troglodyta*. (B) *C. anastomosis*.

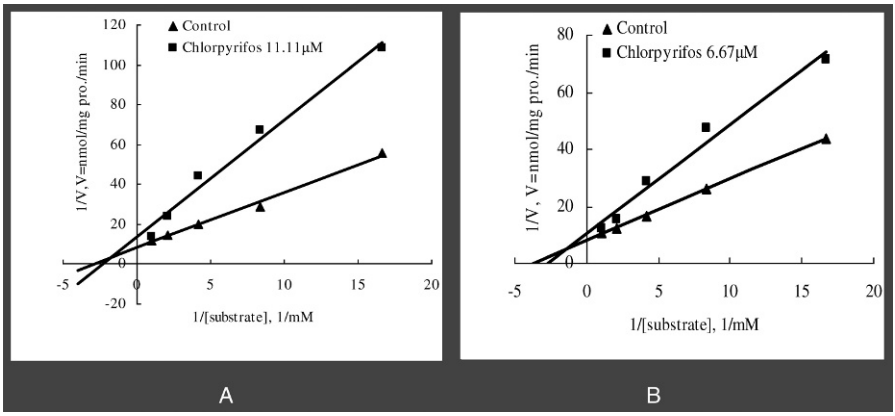


**Fig. 5.** Kinetics of carboxylesterases inhibition by chlorpyrifos in *M. troglodyta* and *C. anastomosis* larvae. (A) *M. troglodyta*. (B) *C. anastomosis*.

*In vitro* inhibition is a useful method for studying the metabolism of xenobiotic compounds catalyzed by carboxylesterases and the carboxylesterase involvement in resistance. In the present study, we found that *in vitro* most of inhibitors tested inhibited carboxylesterases in *M. troglodyta* and *C. anastomosis* larvae at the concentration of 3.3 mM (Table 1), although it is important to note that the degree of inhibition often varied between these two Notodontidae species. This suggests that carboxylesterases in *M. troglodyta* and *C. anastomosis* larvae are qualitatively different in isozyme composition and thus different in sensitivity to inhibitors. Furthermore, *in vitro* inhibition is a useful method for studying the potency of insecticides. We observed that plant allelochemicals (tannic acid and quercetin) are potent inhibitors in our study. Stock et al. (2004) also found that

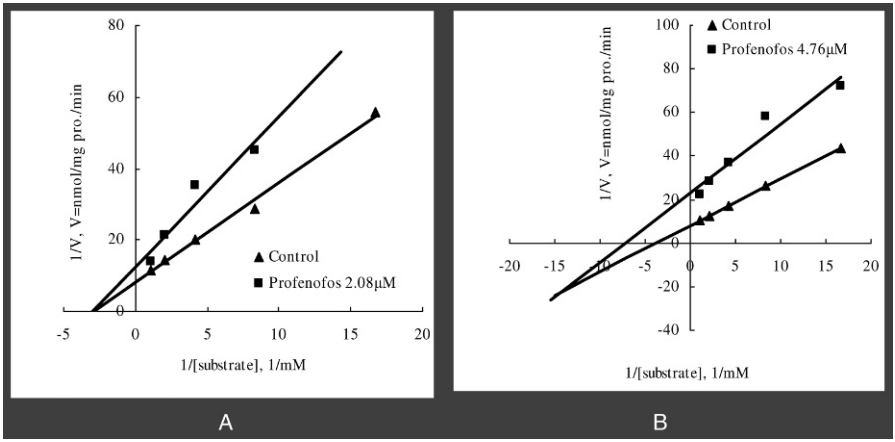


**Fig. 6.** Kinetics of carboxylesterases inhibition by profenofos in *M. troglodyta* and *C. anastomosis* larvae. (A) *M. troglodyta*. (B) *C. anastomosis*.

plant allelochemicals (flavonol compounds of three Mediterranean plants) inhibited the enzymatic activities of both rat intestine and purified porcine liver carboxylesterase. Hopefully, this work will provide the basis for the design of effective, selective carboxylesterase inhibitors and the development of novel candidate insecticides.

Carboxylesterase belong to the group of serine dependent enzymes, some having lipolytic and others proteolytic activities, sharing a catalytic cycle in which the nucleophilic attack on the carbonyl carbon by OH group of serine residue with a concomitant transfer of the proton to histidine residue leads to the formation of a tetrahedral intermediate. Our kinetic analyzes indicated that phoxim competitively inhibited α-NA conjugation activity of carboxylesterases in *C. anastomosis*, which corroborates those reports on other insects. For example, a new flavone glucoside was found to inhibit human carboxylesterase in competitive manner (Djeridane et al. 2008). These results showed that these inhibitors and carboxylesterases have similar substrate hydrolysis preferences. While the noncompetitive inhibition by phoxim in *M. troglodyta* that we observed herein differed from results of Djeridane et al. (2008). This further suggests that carboxylesterases in *M. troglodyta* and *C. anastomosis* larvae are qualitatively different in isozyme composition.

Our results showed that the $I_{50}$ values of phoxim for carboxylesterase activity in *M. troglodyta* and *C. anastomosis* larvae are $2.07 \times 10^{-7}$ M and $5.16 \times 10^{-7}$ M, being the most potent inhibitor. Xu et al. (1999) reported that the $I_{50}$ values of phoxim for carboxylesterases from both the resistant and susceptible strains of *Helicoverpa armigera* (Hübner) were $4.08 \times 10^{-7}$ M and $2.45 \times 10^{-7}$ M, respectively. Furthermore, in the present study, we found that the $I_{50}$ values of chlorpyrifos for carboxylesterase activity in *M. troglodyta* and *C. anastomosis* are $64.85 \times 10^{-7}$ M to $84.39 \times 10^{-7}$ M, respectively. Vioque-Fernández et al. (2007) found that the $IC_{50}$ value of chlorpyrifos was $3 \times 10^{-5}$ M for carboxylesterase activity in *Procambarus clarkii* Crustacea. These results suggested that organophosphate insecticides were effective carboxylesterase inhibitors. Furthermore, different $I_{50}$ values of inhibitors for carboxylesterase activity in *M. troglodyta* and *C. anastomosis* larvae may result in the sensitivity difference of *M. troglodyta* and *C. anastomosis* to pesticides.

## Acknowledgment

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# *In vitro* Inhibition of Carboxylesterases by Insecticides and Allelochemicals in *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae) and *Clostera anastomosis* (L.) (Lepidoptera: Notodontidae)[1]

Fang Tang,[2] Yan-yan Wang,[3] and Xi-wu Gao[4]

J. Agric. Urban Entomol. 25(3): 193–203 (July 2008)

**ABSTRACT** Both *Micromelalopha troglodyta* (Graeser) and *Clostera anastomosis* (L.), are important pests of poplar, and usually coexist during the period of poplar growth in China. The carboxylesterases have been recognized to play an important role in the detoxification of xenobiotics in the two Notodontidae species. *In vitro* inhibitory effects by insecticides and allelochemicals on carboxylesterase activity in *M. troglodyta* and *C. anastomosis* were studied. The results showed that three organophosphates (chlorpyrifos, phoxim and profenofos) were the best inhibitors of the enzymes among all compounds tested in *M. troglodyta* and *C. anastomosis*. Chlorpyrifos inhibited carboxylesterase activity in *M. troglodyta* and *C. anastomosis* to a similar degree, but phoxim and profenofos inhibited to different degrees. Furthermore, kinetic analyses of carboxylesterase inhibition by phoxim, chlorpyrifos and profenofos were also investigated. The results showed that phoxim inhibited carboxylesterase activity with respect to α-naphthyl acetate (α-NA) in a noncompetitive manner in *M. troglodyta*, while the inhibition of phoxim showed competitive to α-NA in *C. anastomosis*. The inhibition of chlorpyrifos showed neither typical competitive nor noncompetitive to α-NA both in *M. troglodyta* and *C. anastomosis*. Profenofos inhibited carboxylesterase activity with respect to α-NA in a noncompetitive manner in *M. troglodyta*, while the inhibition of phoxim showed neither typical competitive nor noncompetitive to α-NA in *C. anastomosis*. These results may contribute to the understanding of the sensitivity difference of *M. troglodyta* and *C. anastomosis* to pesticides, and could provide the basis for integrated pest management of the two Notodontidae species.

**KEY WORDS** *Micromelalopha troglodyta* (Graeser), *Clostera anastomosis* (L.), Lepidoptera, Notodontidae, carboxylesterases, allelochemicals, insecticides

Both *Micromelalopha troglodyta* (Graeser) and *Clostera anastomosis* (L.) are important pests of poplar, causing damage by direct feeding, and usually coexist during the period of poplar growth in China. The control of *M. troglodyta* and *C. anastomosis* is primarily dependent on the application of insecticides. Difference in the efficacy of insecticides between the two Notodontidae species has been

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# A Study of Applied Research Methods and Techniques for Landscape Arthropods: the Crape Myrtle Aphid *Tinocallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae), in Texas[1]

Vassilis A. Vassiliou[3] and Bastiaan "Bart" M. Drees[2]

J. Agric. Urban Entomol. 25(3): 205–221 (July 2008)

**ABSTRACT** Laboratory trials were conducted on crape myrtle aphid *Tinocallis kahawaluokalani* (Kirkaldy) to assess the effectiveness and mode of action of one experimental *Chenopodium*-based botanical (QRD 400 *Chenopodium ambrosioides* var. *ambrosioides*) and retail insecticide products for the control of this insect pest and the effect of these products on different life stages of the multicolored Asian lady beetle *Harmonia axyridis* Pallas. For monitoring aphid densities, two sampling techniques were compared: 1) aphids per 2-cm diameter circular leaf sampling area; and 2) aphid-infested leaves per 5-leaf sample. In both techniques, pre- and post-treatment counts were conducted. Data obtained from both pre- and post-treatment analysis were pooled to provide an average number including analysis of variance with the means separated using Tukey's Honest Significant Difference, at $P \leq 0.05$ level. A linear regression, with 95% mean prediction interval was performed on data assessing various treatment rates of QRD 400.

**KEY WORDS** crape myrtle aphid, insecticides, sampling techniques, *Tinocallis kahawaluokalani*, *Harmonia axyridis*

Crape myrtle, *Lagerstroemia indica* L., is valued as a landscape plant for its prolific summer flowers, heat and drought tolerance, and year-round landscape interest. Flowering begins as early as May in some cultivars and continues into the fall. Each cluster of flowers (or panicle) develops on the tips of new growth and is composed of hundreds of 1- to 2-inch flowers. Color ranges include shades of purple, lavender, white, pink and red, including "true" red, a relatively recent development. Some cultivars have bicolor flowers (two colors on each petal), some cultivars have flower colors that fade with age or certain environmental conditions, and other cultivars have panicles composed of a mix of flower colors.

The crape myrtle aphid (CMA) *Tinocallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae) is the major arthropod pest of crape myrtles, and was apparently introduced into the United States with crape myrtle, its host plant (Mizell & Schiffhauer 1987). CMA is pale yellow in color with winged adults

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having black wings and black protuberances. They primarily are found on undersides of leaves and are particularly attracted to new growth. CMA is not found on any other commonly grown plant. This is the primary aphid species to infest crape myrtle in central Texas (Drees & Jackman 1998).

This species damages crape myrtle by inserting mouthparts into soft tissue and extracting plant sap. Heavy infestations distort leaves and stunt new growth. During feeding, aphids secrete droplets of a sugary solution called "honeydew." Drops of honeydew fall from the aphids onto leaves and stems below. This sugary solution promotes the growth of sooty mold fungi, *Capnodium* sp. Sooty mold appears as a black staining or powdery coating on leaves and stems. The blackened leaves and stems are often the most obvious sign of aphid infestation. The black fungus shades the leaves and interferes with photosynthesis, potentially reducing the long-term vigor of the plant. CMA can reproduce and develop large numbers rapidly. In Texas, populations generally peak between late June and early August (Drees & Jackman 1998). In North Carolina aphid populations remain through October (Baker 1980).

The presence of CMA predators such as *Harmonia* sp. (Coleoptera: Coccinellidae) can dramatically reduce the densities of this aphid species. Predators usually cannot eliminate CMA because their density and distribution depend on biotic (large number versus low number of crape myrtle aphid) and abiotic (rain) factors, resulting in low effect on aphids. Alverson & Allen (1992) reported the emergence of a parasite, *Lysiphlebus testaceipes* (Cresson), from CMA mummies in a greenhouse. Since that time, no other parasites have been reported from CMA in any part of the world. No crape myrtle aphid parasites are found in Southeast Asia, the native habitat of crape myrtle (Mizell et al. 2002). The CMA control is basically relied on both non-chemical and chemical practices. In crowded urban areas, however, the use of insecticidal sprays can be hazardous and people are becoming even more aware of environmental concerns regarding the use of insecticide sprays.

Crape myrtles should be inspected regularly during this period to monitor populations of aphids. Although many predatory insects feed on crape myrtle aphids, they usually cannot control the aphids. Aphid populations can probably be managed if control measures begin by the first week of July. Sprays of insecticidal soaps or horticultural oils are the most environmentally safe pesticides for controlling crape myrtle aphids (Drees 1999). Control of CMA will halt further development of sooty mold.

The main goal of this study was to evaluate the effectiveness of various retail insecticides and application rates for controlling CMA and its non-target beneficial insect predator under laboratory conditions, using different aphid sampling techniques and different insecticide application methods. These methods may allow for rapid assessment of product effectiveness for the ever changing and wide range of products available to consumers annually. For the purposes of this study, we compared sampling methods for evaluating the aphid densities before and following insecticide application. The contact effect of the insecticidal soap applied on the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, larvae and adults was also studied. Results of conducting multiple sampling methods simultaneously are intended to help other applied researchers better understand various methods and techniques available to design, conduct and evaluate insecticide effectiveness or efficacy studies.

## Materials and Methods

**Location and material.** All tests were carried out in the laboratory at the Center for Urban & Structural Entomology, Department of Entomology, Texas A&M University, College Station, Texas. Both aphid-infested and non-infested crape myrtle shoots from heavily infested landscape shrubs (unknown variety), as well as lady beetle adults and larvae were collected from the Riverside Campus of the Texas A&M University, Brazos County, TX during July and August 2007.

**Experimental techniques and application**. *Trial 1. Treatments with shoots.* Freshly cut 20–35 cm long crape myrtle shoots (17 July 2007 and 26 July 2007) containing an average of 20–35 leaves with visible aphid infestation collected from a single shrub were placed in containers with soil and water and carried to the laboratory. Containerized shoots were maintained indoors at room temperature t = 24°C ± 1°C, and RH = 65%. Pre-treatment counts were made at the above-mentioned dates for: a) the absence/presence of aphids on a 5-leaf sample and, b) the number of wingless adults and nymphs in a 2-cm diameter circle sampling area. In both cases, 5 leaves on each shoot were randomly selected and marked with a Sharpie® pen for post-treatment counts. For the 17 July 2007 and 26 July 2007 tests, 5 and 7 treatments were arranged including an untreated (water spray, only) control. Containerized shoots were arranged in 4 replications or treatment blocks to array aphid numbers from highest to lowest based on pre-treatment aphid density from 2-cm diameter circular sampling area method results, with the first replicate or block having the highest levels of aphids per sample and forth replicate having the lowest levels (Reinert 1976). Treatments (Tables 1 and 2) were assigned randomly to containerized shoots within each replicate and adjusted to minimize pre-treatment aphid density mean values between treatments. Each treatment was then applied to 1 shoot per treatment for each replicate. Insecticides were applied on the same day outside the laboratory using 250 ml of insecticide solution for per four shoots. All insecticides were applied using a hand sprayer "Polyspray 2" (Geiger Enterprices, P.O. Box 285 Harleysville, PA 19438) model 4075 with a 1 mm diameter regular nozzle for foliar applications and 1-liter capacity. Post-treatment aphid counts were performed at 24 h of exposure to treatments.

*Trial 2. Laboratory trial using the leaf dip technique.* For this trial, freshly cut crape myrtle shoots of about 20–35 cm long, and containing 20–35 leaves (26 July 2007) were cut in the field, immediately placed into pots with soil and water, and transferred to the laboratory. Only healthy leaves with no symptoms of powdery mildew were selected. The leaves were cut with a stem of about 1.5 cm in length and washed with tap water in order to remove any aphids and dirt, and let to dry for 30 min using a fan. A total of 28 leaves were used in 7 treatments (Table 2) and 4 replications (1 leaf for each treatment). All replications included 6 treatments with insecticides and 1 untreated (water only) control. The solutions were prepared in Petri dishes where an amount of 30 ml of each insecticide was used. Each leaf was dipped in the respective insecticide for 5 sec. All the leaves were let to dry for 45–60 min using a fan to accelerate the drying process. After they dried, each leaf stem was wrapped in a piece of cotton and placed in a 1.5 mL tube with water. Every tube top was wrapped with parafilm in order to avoid the water to flow. Crape myrtle aphids were maintained indoors at room temperature t = 24°C ± 1°C, and RH = 65% on freshly cut infested shoots. Twenty nymphs were transferred onto each

**Table 1. Insecticides applied in laboratory trials. 18 July 2007, College Station, Brazos Co., TX.**

| Commercial name | Company | Active ingredient | Label dosage (fl. oz/gal.) | Dosage used | Mode of action | Toxicity to beneficial insects |
|---|---|---|---|---|---|---|
| QRD 400 | AgraQuest Inc., 1530 Drew Avenue, Davis, CA 95616-1272 | *Chenopodium ambrosioides* var. *ambrosioides* | 2.5 | 3[a] | Contact | NO |
| insecticidal soap® | Woodstream Corp., 69 North Locust str, Lititz, PA 17543 | potassium salts of fatty acids 49.52% | 2.5 | 5[a] | Contact | Bubble bees, syrphids and lady beetle adults relatively unaffected |
| Rose defense® Ready -To -Use | Green light Co., P.O. Box 17985, San Antonio, TX 78217-0985 | clarified hydrophobic extract of neem oil 0.9% | – | 250[b] | Contact | NO |
| MAX® Ready -To -Use | ORTHO Group, P.O. Box 190, Marysville, OH 43040 | esfenvalerate 0.0033% | – | 250[b] | Contact | YES |

[a]ml of insecticide used in 250 ml of water.
[b]Amount of insecticide Ready-To-Use.

**Table 2. Insecticides applied in leaf dip laboratory tests and shoot sprays. 24 July 2007, College Station, Brazos Co., TX.**

| Commercial name | Company | Active ingredient | Label dosage (fl. oz/gal.) | Mode of action | Toxic to beneficial insects |
|---|---|---|---|---|---|
| Biorganic® Ready-To-Use | Green light Co., P.O. Box 17985, San Antonio, TX 78217-0985 | Thyme oil 0.33%, Clove oil 0.33%, Sesame oil 0.33% | – | Contact | NIA[b] |
| Garden Safe™ Ready-To-Use | W. Neudorff, GmbH KG, Germany | Pyrethrins 0.01%, Canola oil 1.00% | – | Contact | YES |
| Garden Safe™ Ready-To-Use | Distributed by Schultz Co., P.O. Box 4406 Bridgeton, MO 63044-0406 | Pyrethrins 0.02%, piperonyl butoxide, Technical 0.20% | – | Contact | YES |
| Organocide™ Ready-To-Use | Organic Laboratories, Inc. P.O. Box 1931 Stuart, FL 34995 | Sesame oil 0.10% | – | Contact | NIA[b] |
| All Seasons® | Bonide Products, Inc., 6301 Suttiff Road, Oriskany, NY 13424 | Petroleum oil (Superior paraffinic type U.R. Min 92%) | 2.5[a] | Contact | NIA[b] |
| Bayer Advanced™ Ready-To-Use | BayerCropScience LP, P.O. Box 12014, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709 | β-cyfluthrin 0.0015% imidacloprid 0.0120% | – | Contact | YES |

[a]1 ml of the product was added to 50 ml of water.
[b]No Information Available.

treated leaf surface using a fine paintbrush. Once aphids were on the leaf surface, each tube (with the leaf and the nymphs) was placed in a Petri dish where the upper and lower disks were wrapped with parafilm in order to avoid aphids from a probable escape. Post-treatment counts were made after 24 h.

**Calculating "percent control".** Using the Abbott's (A) and Henderson—Tilton's formulas (B) we calculated the effectiveness of the applied insecticides compared to the control (Trials 1 and 2).

$$\text{(A) Corrected \%} = 1 - \frac{\text{n in T after treatment}}{\text{n in Co after treatment}} * 100$$

Where: n = insect population, T = treated, Co = control

$$\text{(B) Corrected \%} = 1 - \frac{\text{n in Co before treatment} * \text{n in T after treatment}}{\text{n in Co after treatment} * \text{n in T before treatment}} * 100$$

Where: n = insect population, T = treated, Co = control

*Trial 3. Effect of insecticidal soap on predators.* For this test, multicolored Asian lady beetle *Harmonia axyridis* larvae and adults were used. Adults and larvae were collected from CMA infested shoots, and then put in clear plastic "zip" lock bags and carried to the laboratory. On 1 August 2007 (Test 1), 54 adults were collected and for the 6 August test, 90 adults and 90 larvae (6 adults and 6 larvae per treatment) were collected (Tests 2 and 3, conducted on adults and larvae separately). Three treatments (Table 3) and 3 replications were arranged for these tests. In both trials, the treatments were applied in the laboratory at the same day at 3:00 pm on Whatman® filter paper circles (150 mm diameter), using the "Polyspray 2" hand sprayer. Fifty mL of each solution were required to spray the filter paper and the insects with each treatment. In all treatments, treated filter paper was placed in each clean Petri dish. No food was provided to adults and larvae. Adults and larvae were immobilized using $CO_2$ in order to ease the transferring process. In all cases, the hand sprayer was washed twice before was used for the following application. Adult and larvae mortality counts were performed at 0.5, 24 and 48 h of exposure to treatments.

*Trial 4. QRD 400 rate tests on crape myrtle aphids.* The efficacy of the botanical product QRD 400 (*Chenopodium ambrosioides* var. *ambrosioides*) on the crape myrtle aphid-infested shoots was evaluated beginning 16 August 2007. For this trial, 4 treatments (rates) of the product and a control (water) were tested in 4 replications (Table 8). Mortality was documented 24 h post-treatment.

**Data analysis.** Treatment means were subjected to an analysis of variance (ANOVA), with the means separated using Tukey's Honest Significant Difference, at $P \leq 0.05$ (SPSS for Windows, Version 14.0) (Trials 1, 2, 3 and 4). In addition, linear regression analysis was performed (SPSS for Windows, Version 14.0) at $P \leq 0.05$ (Trial 4).

## Results

**Trial 1. Treatments with shoots.** Pre-treatment counts conducted, 18 July 2007, proved to be a powerful tool in estimating the aphid numbers on

**Table 3. Treatments used in trials for the evaluation of the effect of insecticidal soap on two life stages of the multicolored Asian lady beetle *Harmonia axyridis* Pallas.**

| Date | Life stage used | Treatments | No. of insects/ treatment |
|---|---|---|---|
| 1 August 2007 | Adult | 1. Control (water) – adults were released after the treated filter paper was dried | 6 |
| | | 2. Insecticidal soap – adults were released after the treated filter paper was dried | 6 |
| | | 3. Insecticidal soap – adults and filter paper were sprayed at the same time | 6 |
| 6 August 2007 | Adult | 1. Control (water) – adults were released after the treated filter paper was dried | 10 |
| | | 2. Insecticidal soap – adults were released after the treated filter paper was dried | 10 |
| | | 3. Insecticidal soap – treated adults were released on a treated dried filter paper | 10 |
| 6 August 2007 | Larva | 1. Control (water) – larvae were released after the treated filter paper was dried | 10 |
| | | 2. Insecticidal soap – larvae were released after the treated filter paper was dried | 10 |
| | | 3. Insecticidal soap – treated larvae were release on a treated dried filter paper | 10 |

the 5 marked sampled leaves. No significant differences were detected in mean aphid numbers in pre-treatment analysis (Table 4) using results from the 2-cm diameter circle sampling technique (df = 4; $F = 0.011$; $P \leq 0.05$). Post-treatment analysis revealed that all the insecticides applied on shoots showed similar effectiveness on wingless crape myrtle aphids compared to the untreated (water, only) control, with no significant differences were observed between the insecticide treatments using any method of mean separation methods.

Using the Abbott's formula for percent control counting (Table 6a) among the insecticides used in trials es-fenvalerate killed 99.3% of nymphs followed by the insecticidal soap (97.8%), the QRD 400 (97%), and Neem oil (91.8%). These insecticides gave also similar results where the number of aphid-infested leaves per 5-leaf sample was used.

**Table 4. Mean number of crape myrtle aphids per 2-cm diameter circle sampling area per leaf and number of aphid-infested leaves per 5-leaf sample, College Station, Brazos Co., TX, applied 18 July 2007.**

| Treatments | Aphids/2-cm sample[a] Pre-treatment | Aphids/2-cm sample[a] 24 h Post-treatment | Infested leaves/5[a] Pre-treatment | Infested leaves/5[a] 24 h Post-treatment |
|---|---|---|---|---|
| Untreated (water) | 29.2 a[a] | 13.5 a[a] | 5.0 a[a] | 4.5 a[a] |
| QRD 400 | 27.0 a | 0.4 b | 4.5 a | 1.5 b |
| Insecticidal soap | 29.3 a | 0.3 b | 4.3 a | 1.5 b |
| Neem oil | 29.9 a | 1.1 b | 4.5 a | 3.0 ab |
| Esfenvalerate | 29.5 a | 0.1 b | 4.5 a | 0.3 b |
| df = 4 | df = 4 | df = 4 | df = 4 | df = 4 |
| *F* value | 0.011 | 6.183 | 0.383 | 5.784 |
| *P* value | 1.000 [b] | 0.004 | 0.812 [b] | 0.005 |
| Mean Sq. | 5.357 | 137.558 | 0.330 | 10.700 |

[a]Means in columns followed by the same letter are not significantly different using Analysis of Variance (ANOVA), with means separated using Tukey's Honest Significant Difference at $P \leq 0.05$ level.
[b]Not significantly different.

No significant differences were observed in mean aphid numbers in pre-treatment counts conducted with different set of product treatments initiated on 27 July 2007 (Table 5) also using the 2-cm diameter circle sampling technique (df = 6; $F = 0.067$; $P = 0.999$). Again, post-treatment analysis revealed significant differences between the insecticide treatments and the untreated control (df = 6; $F = 4.455$; $P = 0.005$), but no differences were detected between the insecticide treatments.

Percent control counting using the Abbott's formula showed that β-cyfluthrin plus imidacloprid and Pyrethrins plus piperonil butoxide (synergist) provided 100% aphid mortality followed by thyme oil plus clove oil plus sesame oil with 99.3% mortality (Table 6b).

No significant differences in aphid nymphs means were detected in pre-treatment counts (df = 6; $F = 21.071$; $P = 0.00$), where the aphid-infested leaves per 5-leaf sample (presence/absence of aphids) was used. Statistical analysis showed differences in post-treatment counts using Tukey's means separations (df = 4; $F = 6.183$; $P = 0.004$; Mean sq. = 137.558 and df = 4; $F = 5.784$; $P = 0.005$; Mean sq. = 10.700) (Table 4 and 5, respectively). Among the insecticides used in these treatments, β-cyfluthrin plus imidacloprid and pyrethrins plus piperonil butoxide provided 100% aphid mortality (Table 6b).

**Trial 2. Laboratory tests using the leaf dip technique.** The post-treatment analysis (27 July 2007) showed differences between insecticide treatments and the untreated control but no significant differences were observed between the insecticide treatments (df = 6; $F = 113.465$; $P = 0.00$) (Table 5). These results reveal that all insecticides used in these treatments were effective against crape myrtle aphid, and that these ingredients appear to have activity causing mortality to non-sprayed aphids as residual contact insecticides

**Table 5. Mean number of crape myrtle aphids per 2-cm diameter circle sampling area per leaf, number of aphid-infested leaves per 5-leaf sample, and number of dead aphids using the leaf dip technique. College Station, Brazos Co., TX, applied 27 July 2007.**

| Treatments | Aphids/2-cm sample[a] Pre-treatment | Aphids/2-cm sample[a] 24 h Post-treatment | Infested leaves/5[a] Pre-treatment | Infested leaves/5[a] 24 h Post-treatment | 20 aphids /leaf. Post-treatment dead nymphs[a] |
|---|---|---|---|---|---|
| Untreated (water) | 43.2 a[a] | 14.4 a[a] | 5.0 | 0.9 a[a] | 18.3 a[a] |
| sesame oil | 38.3 a | 0.35 b | 5.0 | 0.3 bc | 0.5 b |
| β-cyfluthrin + imidacloprid | 40.7 a | 0.00 b | 5.0 | 0.0 c | 0.5 b |
| Pyrethrins + piperonil butoxide | 45.3 a | 0.00 b | 5.0 | 0.0 c | 1.0 b |
| Petroleum oil | 39.3 a | 1.65 b | 5.0 | 0.5 b | 0.5 b |
| Pyrethrins + canola oil | 38.9 a | 0.25 b | 5.0 | 0.1 c | 0.3 b |
| thyme oil + clove oil + sesame oil | 37.8 a | 0.10 b | 5.0 | 0.1 c | 0.3 b |
| df = 6 | df = 6 | df = 6 | - | df = 6 | df = 6 |
| *F* value | 0.067 | 4.455 | - | 21.071 | 113.865 |
| *P* value | 0.999 [b] | 0.005 | - | 0.000 | 0.000 |
| Mean Sq. | 31.023 | 113.465 | 0.000 | 0.421 | 180.286 |

[a]Means in columns followed by the same letter are not significantly different using Analysis of Variance (ANOVA), with means separated using Tukey's Honest Significant Difference at $P \leq 0.05$ level.
[b]Not significantly different.

**Table 6a. Insecticide percent control calculations using Abbott's and Henderson-Tilton's formulas on aphid-infested leaf samples. 18 July 2007.**

| | Abbott's, % | | Henderson's-Tilton's, % | | % from pre-treatment mean | |
|---|---|---|---|---|---|---|
| Treatment | 2 cm diameter circle | Per 5-leaf sample | 2 cm diameter circle | Per 5-leaf sample | 2 cm diameter circle | Per 5-leaf sample |
| Untreated (water) | - | - | - | - | 53.8 | 10.0 |
| QRD 400 | 97.0 | 66.7 | 96.8 | 62.9 | 98.5 | 66.7 |
| insecticidal soap | 97.8 | 66.7 | 97.8 | 61.2 | 98.9 | 65.1 |
| Neem oil | 91.8 | 33.3 | 92.0 | 25.3 | 96.3 | 33.3 |
| Esfenvalerate | 99.3 | 93.3 | 99.3 | 92.6 | 99.7 | 93.3 |

**Table 6b. Insecticide percent control calculations using Abbott's and Henderson-Tilton's formulas on aphid-infested leaf samples. 27 July 2007.**

| | Abbott's, % | | Henderson's-Tilton's, % | | % from pre-treatment mean | |
|---|---|---|---|---|---|---|
| Treatment | 2-cm diameter circle | Per 5-leaf sample | 2-cm diameter circle | Per 5-leaf sample | 2-cm diameter circle | Per 5-leaf sample |
| Untreated (water) | 0.0 | 0.0 | 0.0 | 0.0 | 66.7 | 82.0 |
| Sesame oil | 97.6 | 66.7 | 97.3 | 66.7 | 99.1 | 94.0 |
| β-cyfluthrin + imidacloprid | 100.0 | 100.0 | 97.3 | 100.0 | 100.0 | 100.0 |
| Pyrethrins + piperonil butoxide | 100.0 | 100.0 | 94.5 | 100.0 | 100.0 | 100.0 |
| Petroleum oil | 88.5 | 44.4 | 97.3 | 44.4 | 95.8 | 90.0 |
| Pyrethrins + canola oil | 98.3 | 88.9 | 98.4 | 88.9 | 99.3 | 98.0 |
| Thyme oil + clove oil + sesame oil | 99.3 | 88.9 | 98.4 | 88.9 | 99.7 | 98.0 |

on the treated leaf surface, and not requiring direct application of treatments to aphids.

**Trial 3. Effect of insecticidal soap on predators**. *Test 1: 1 August 2007.* After 24 h of exposure to the treated filter paper all the *Harmonia* adults were alive revealing that the insecticidal soap didn't show to have any contact effect on beetles (Table 7) (df = 2; $F$ = 0.0; $P$ = 0.0). No statistically significant differences were observed in adult mortality across the treatments. However, increase in adult mortality was observed 48 h after exposure of adults on the treated filter paper that had remained moist, being significantly greater than mortality on dried insecticidal soap and water treated filter paper. Thus, toxic effects of soap appear in a manner not associated with desiccation of the treated insects.

*Test 2 and 3: 6 August 2007.* An initial knockdown of larvae was observed (Table 8, 0.5 h) whereby they were all observed to stop moving and appeared dead immediately after the application of the soap spray. Over the next 2–15 h a significant number of larvae recovered. No statistically significant differences (df = 2; $F$ = 0.0; $P$ = 1.0) were observed in the 24 h exposure of larvae to all the three treatments. No statistically significant differences in adult or larval *Harmonia* mortality (df= 2; $F$ = 0.0; $P$ = 0.0) have been observed in the 24 h exposure of treated adults or exposure of adults to treated filter paper (Table 7b). Increase in adult mortality with statistically significantly differences was observed in the 48 h exposure of treated adults on the treated filter paper. However, this mortality was evidently caused by a factor other than the soap treatment as it increased in the untreated (water, only) control. Predation of larger larvae on small larvae was observed and likely affected the outcome of this trial.

**Trial 4. QRD 400 rate tests on crape myrtle aphids.** The ANOVA showed statistically significant differences between QRD 400 different rates and the control in post-treatment counts using the 2-cm diameter circle sampling area (df = 4; $F$ = 9.424; $P$ = 0.001; Mean sq. = 165.038) but no statistically significant differences between QRD 400 rates were observed (Table 9). Post-treatment

**Table 7. Ladybird *Harmonia axyridis* adult mortality after 0.5 h, 24 h and 48 h of exposure to treatments. 1 August 2007.**

| | Time of exposure | | |
|---|---|---|---|
| Treatment | 0.5 h | 24 h | 48 h[a] |
| Control (water) - filter paper dried | 0.0 | 0.0 | 0.0 a[a] |
| Insecticidal soap - treated filter paper dried | 0.0 | 0.0 | 0.7 a |
| Insecticidal soap - treated filter paper not dried - treated adults | 0.0 | 0.0 | 6.0 b |
| df = 2 | df = 2 | df = 2 | df = 2 |
| $F$ value | - | - | 292.400 |
| $P$ value | - | - | 0.000 |
| Mean sq. | - | - | 32.444 |

[a]Means in columns followed by the same letter are not significantly different using Analysis of Variance (ANOVA), with means separated using Tukey's Honest Significant Difference at $P \leq 0.05$ level.

**Table 8. Ladybird *Harmonia axyridis* adult and larvae mortality after 0.5 h, 24 h and 48 h of exposure to treatments. 6 August 2007.**

| | Time of exposure | | | | | |
|---|---|---|---|---|---|---|
| | Adults | | | Larvae | | |
| Treatment | 0.5 h | 24 h | 48 h[a] | 0.5 h[a] | 24 h | 48 h |
| Control (water) - filter paper dried | 0.0 a | 0.0 | 1.0 a | 0.3 a | 1.3 a | 1.0 a |
| Insecticidal soap - treated filter paper dried | 0.0 a | 0.0 | 0.0 b | 0.0 a | 1.3 a | 0.0 a |
| Insecticidal soap - treated filter paper dried – treated adults and larvae | 0.7 a | 0.0 | 1.3 a | 2.7 b | 1.3 a | 1.0 a |
| df = 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| *F* value | 4.000 | - | 13.000 | 28.500 | 0.000 | 1.500 |
| *P* value | 0.079 [b] | - | 0.007 | 0.001 | 1.000 [b] | 0.296 [b] |
| Mean sq. | 0.444 | - | 1.444 | 6.333 | 0.000 | 1.000 |

[a]Means in columns followed by the same letter are not significantly different using Analysis of Variance (ANOVA), with means separated using Tukey's Honest Significant Difference at $P \leq 0.05$ level.
[b]Not significantly different.

**Table 9. QRD 400 rate tests on crape myrtle aphid. 16 August 2007.**

| Treatment | ml/250 ml of water | Aphids/2-cm sample[a] Pre-treatment | Aphids/2-cm sample[a] 24 h Post-treatment | Infested leaves/5[a] Pre-treatment | Infested leaves/5[a] 24 h Post-treatment |
|---|---|---|---|---|---|
| Control (water) | 250 | 51.7 a | 14.9 a | 5.0 | 0.9 a[a] |
| QRD 400, 0.25% | 0.6 | 49.3 a | 0.7 b | 5.0 | 0.3 b |
| QRD 400, 0.5% | 1.25 | 49.3 a | 0.8 b | 5.0 | 0.4 b |
| QRD 400, 1.0% | 2.5 | 48.8 a | 1.1 b | 5.0 | 0.3 b |
| QRD 400, 2.0% | 5.0 | 52.2 a | 0.0 b | 5.0 | 0.0 b |
| df = 4 | | df = 4 | df = 4 | df = 4 | df = 4 |
| *F* value | | 0.10 | 9.424 | - | 8.760 |
| *P* value | | 1.000 [b] | 0.001 | - | 0.001 |
| Mean sq. | | 1027.518 | 165.038 | - | 0.050 |

[a]Means in columns followed by the same letter are not significantly different using Analysis of Variance (ANOVA), with means separated using Tukey's Honest Significant Difference at $P \leq 0.05$ level.
[b]Not significantly different.

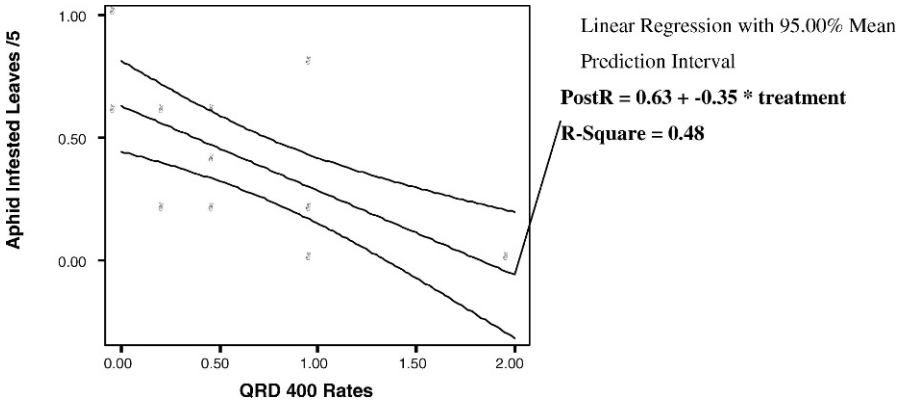


**Fig. 1.** Effect of QRD 400 concentrations on the survivorship of crape myrtle aphids using the aphid- infested leaves per 5-leaf sample.

analysis using the aphid-infested leaves per 5-leaf sample showed statistically significant differences between QRD 400 rates and the control (df = 4; $F$ = 8.760; $P$ = 0.001; Mean sq. = 0.050) but no differences were observed between the rates using Tukey's means separations.

There was a linear relationship ($r^2$ = 0.48) between QRD 400 concentrations and aphid survivorship using the aphid-infested leaves per 5-leaf sample ($R^2$ Adjusted = 0.447; $F_{1,18}$ = 16.333; $P$ = 0.001; Mean sq. = 1.190), where post-treatment counts (PostR) = 0.63 + −0.35 * Treatment (Fig. 1). Linear relationship was also observed ($r^2$ = 0.23) between QRD 400 rates and number of live aphids using the 2-cm diameter circle sample area ($R^2$ Adjusted =0.185; $F_{1,18}$ = 5.310; $P$ = 0.033; Mean sq. = 210.222), where post-treatment counts (PostC) = 6.92 + −4.59 * Treatment (Fig. 2). The $r^2$ for the regression analysis of data generated by the aphid-infested leaves per 5-leaf sampling method was higher than that generated by the 2-cm diameter circle sample area method.

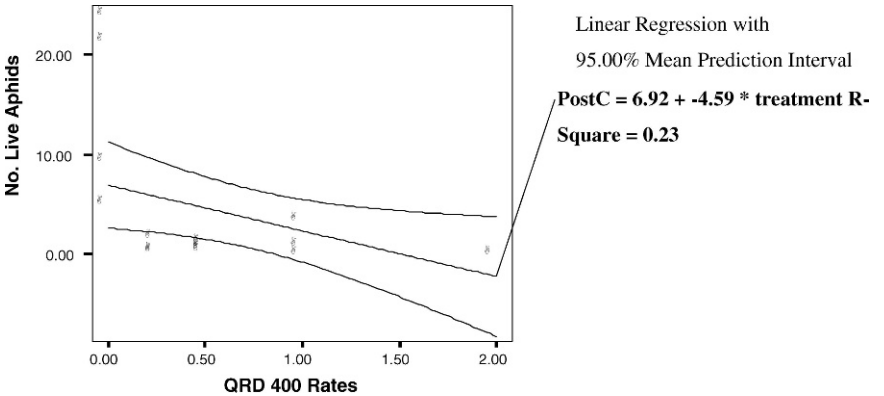


**Fig. 2.** Effect of QRD 400 concentrations on the survivorship of crape myrtle aphid using the 2-cm diameter circle sample area per 5-leaf sample.

## Discussion

*Insecticides evaluated.* Synthetic insecticides remain the main products for controlling various insect pests on many agricultural crops, ornamental landscaping plants, and home gardens. However, many botanical and other industrial compounds are available for outdoors and greenhouse use. These products appear to be environmentally friendly and some can be considered organic products for control of insect pests, particularly small soft-bodied arthropods. Most traditional insecticide products are registered for use in the U.S. by the Environmental Protection Agency (EPA). However, a recently new provision in the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) called 25b, allows for development and sale of products with insecticidal claims that are exempt from registration by EPA because their ingredients are "of food grade" materials. Many of these products have not been evaluated by the Texas AgriLife Extension Service or elsewhere by third party research personnel.

Most of the insecticides tested in our trials have direct contact toxic effects requiring them to be applied to the arthropod pest itself or to the surface on which a pest crawls, and at the same time, they appeared to have low toxicity to beneficial insects, humans and environment. Only two of the tested contact insecticides have systemic effects on the insect nervous system: Bayer Advanced™ Rose and Flower Insect Killer contains β-cyfluthrin, a synthetic pyrethroid ingredient, plus the systemic ingredient imidacloprid, a neonicotinoid insecticide; Ortho® Max Garden and Landscape Insect Killer, contains the systemic pyrethroid ingredient, es-fenvalerate. In addition, imidacloprid has systemic properties within plant tissue.

Insecticidal soap products have often been used because they generally leave minimal residues, are less toxic to beneficial insects, are short-lived in the environment because they degrade rapidly, and they are relatively non-toxic to the user. Insecticidal soap is used to control many soft-bodied insect and mite pests including aphids, scales, mealybugs, and the two-spotted spider mite. Small soft-bodied pests including CMA are most susceptible to soap applications. The mode of action of insecticidal soaps is not well understood. The cuticular wax layer on arthropods sprayed with soap solution is thought to become disrupted, causing loss of water and desiccation. In addition, the soap solution can enter the respiratory system through the spiracles causing suffocation and possibly cell death. The results from our trials showed high effect of insecticidal soap on CMA (97.8%).

Insecticidal soaps, generally, have minimal activity on beetles and other hard-bodied insects although this is not always true, as soaps have been shown to kill hard-bodied insects such as cockroaches (Szumlas 2002). Soaps are thought to be effective only when insects come into direct contact with the spray. Dried residues on plant surfaces have minimal insecticidal activity as soap residues degrade rapidly. Insecticidal soap showed to have no contact effect on multicolored Asian lady beetle *Harmonia axyridis* adults and larvae, after 24 h of exposure to soap. Once the insecticidal soap spray had dried, there was no residual activity, and if an insect has not been coated with the spray, walking over or ingesting any material that has been treated with soap will not affect it. Interestingly, mortality was observed when adult beetles were sprayed on wet filter paper, indicating that mortality can occur due to some other mechanism than desiccation such as suffocation or cytotoxicity.

QRD 400 is a new botanical product derived from *Chenopodium ambrosioides* var. *ambrosioides* L., an indigenous plant to Central and South America, and appears to have insecticidal, acaricidal and fungal properties. This botanical insecticide is found to be a good alternative for managing soft-bodied insect pests of various greenhouse and outdoors crops such as green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae), western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), citrus mealybug, *Planococcus citri* (Risso) (Homoptera: Pseudococcidae), two- spotted mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae), and fungus gnats, *Bradysia* spp. (Diptera: Sciaridae) (Chiasson et al. 2004, Cloyd & Chiasson 2007).

Although in our trials no significant differences were observed between rates of the *Chenopodium*-based botanical QRD 400 using Analysis of Variance, it caused high mortality to CMA, especially the 2% rate.

*Plot design considerations.* Designing applied research suitable for statistical analysis to document the effectiveness of insecticides of control of landscape pests can be problematic. In this instance, we wished to evaluate a number of "new" insecticide products available for treatment of "aphids" on ornamental and flowering landscape pests. However, we were able to locate an infestation of crape myrtle aphids on about five shrubs on the Riverside Campus of the Texas A&M University. Thus, establishing a replicated trial of two or more treatments was not feasible because there were too few shrubs for replicate treatments. Thus, the decision was made to use containerized shoots as treatment "plots". Shoots cut from the shrubs and brought into the laboratory, however, decline rapidly. Winged aphids fly away and wingless aphids decline as plant materials perishes. Thus, untreated control (water only treatment) mortality was anticipated over a short period of time. This, only 24 h post-treatment assessments were deemed feasible. However, use of this method allowed for numerous treatments to be assessed rapidly in a complete randomized block design.

To minimize and/or eliminate any pre-treatment mean differences in aphid population density assessments between replicates, the method used by Reinert (1976) was employed. Avoiding significant pre-treatment differences between means allowed for comparison between treatments in post-treatment evaluation to be accomplished without manipulating data further. Regardless, minor numerical differences in pre-treatment data can affect some methods used to calculate percent reduction (percent control, percent mortality) values.

*Sampling methods.* Aphids have high reproductive potential (parthenogenesis) leading to extremely high population densities. CMA is host-specific to crape myrtle varieties that do not have resistance to this pest. Their population densities and distribution can be documented using various sampling techniques that can be useful evaluating the efficacy of various insecticides over time. CMA population density and distribution on their host crape myrtles vary over time, by variety, and location. Pre-treatment counts of infested sampled crape myrtle shoots proved to be a helpful tool in assessing the aphid population density per sampling unit. Using the 2-cm diameter circular leaf sampling area technique provided more precise and real evaluation of the population density on the sampled leaves, but it required much more time.

The aphid- infested leaves per 5-leaf sample technique required less time in recording aphid presence or absence on leaves, but failed to show more subtle

differences in post-treatment mortality differences between treatments caused by insecticides (Tables 1 and 2). In this case, a leaf was considered occupied when even one aphid nymph was present, reducing the estimation of the actual aphid density in the sample. Conversely, in instances where low population densities occur in such research, sampling more plant material more quickly can overcome low counts or zero data points (non-infested leaves) in pre-treatment evaluations. Pre-treatment results obtained from both sampling techniques used here contributed in blocking from the highest to the lowest aphid numbers or infested leaf numbers and assigning treatments, providing a pre-count uniformity between treatment means.

*Statistical methods.* The purpose of these experiments was to evaluate the effectiveness of various insecticides and insecticide rates on CMA, and compare the mean of each of these treatment group results with the mean of an untreated control (water spray only) treatment group. In this case, an Analysis of Variance was used in order to separate the means. In our trials, we have employed the Tukey's Honest Significant Difference (Tables 4, 5 and 7).

Linear Regression was also used to analyze the relationship between aphid mortality and QRD 400 at different rates. The goal here was to adjust the values of slope and intercept to find the line that best predicts Y from X, and minimize the sum of squares of the vertical distances of the points from the line. The higher the dose used the more the aphids were killed. Because the linear regression equation generated was significant (Fig. 1 and 2), any point along the line is significantly different from any other point along this line.

*Methods of calculating percent control.* Two methods of calculating percent control were used to calculate the difference caused by the application of insecticides compared to an untreated (water spray only) control treatment group: Abbott's; and Henderson's –Tilton's formulas. Unlike Abbott's formula, the Henderson–Tilton's formula takes into consideration pre-treatment sampling differences between treatment groups and calculates the proportion of animals (i.e., insects) dying in a trial between treatment and untreated control groups and compensated for possible control group mortality occurring by the post-treatment assessment.

The results of these experiments conducted in a laboratory environment, using various methods used for sampling and analysis, yield some baseline data for conducting further field experiments. Some sampling methods used, such as the 5-leaf sample can save time, can allow for increase a volume of plant material sampled, but reduce the resolution of population level estimates documented. Calculation results for "percent control" can also differ and depend on method chosen. Awareness of these issues should be helpful to anyone conducting applied research to document treatment efficacy for candidate insecticide products.

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# A Study of Applied Research Methods and Techniques for Landscape Arthropods: the Crape Myrtle Aphid *Tinocallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae), in Texas[1]

Vassilis A. Vassiliou[3] and Bastiaan "Bart" M. Drees[2]

J. Agric. Urban Entomol. 25(3): 205–221 (July 2008)

**ABSTRACT** Laboratory trials were conducted on crape myrtle aphid *Tinocallis kahawaluokalani* (Kirkaldy) to assess the effectiveness and mode of action of one experimental *Chenopodium*-based botanical (QRD 400 *Chenopodium ambrosioides* var. *ambrosioides*) and retail insecticide products for the control of this insect pest and the effect of these products on different life stages of the multicolored Asian lady beetle *Harmonia axyridis* Pallas. For monitoring aphid densities, two sampling techniques were compared: 1) aphids per 2-cm diameter circular leaf sampling area; and 2) aphid-infested leaves per 5-leaf sample. In both techniques, pre- and post-treatment counts were conducted. Data obtained from both pre- and post-treatment analysis were pooled to provide an average number including analysis of variance with the means separated using Tukey's Honest Significant Difference, at $P \leq 0.05$ level. A linear regression, with 95% mean prediction interval was performed on data assessing various treatment rates of QRD 400.

**KEY WORDS** crape myrtle aphid, insecticides, sampling techniques, *Tinocallis kahawaluokalani*, *Harmonia axyridis*

Crape myrtle, *Lagerstroemia indica* L., is valued as a landscape plant for its prolific summer flowers, heat and drought tolerance, and year-round landscape interest. Flowering begins as early as May in some cultivars and continues into the fall. Each cluster of flowers (or panicle) develops on the tips of new growth and is composed of hundreds of 1- to 2-inch flowers. Color ranges include shades of purple, lavender, white, pink and red, including "true" red, a relatively recent development. Some cultivars have bicolor flowers (two colors on each petal), some cultivars have flower colors that fade with age or certain environmental conditions, and other cultivars have panicles composed of a mix of flower colors.

The crape myrtle aphid (CMA) *Tinocallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae) is the major arthropod pest of crape myrtles, and was apparently introduced into the United States with crape myrtle, its host plant (Mizell & Schiffhauer 1987). CMA is pale yellow in color with winged adults

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# Evaluation of Chemical Control Strategies for *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) in South Carolina State Park Campgrounds[1]

Brittany R. Ellis,[2] Eric P. Benson,[2] Patricia A. Zungoli,[2] and William C. Bridges, Jr.[3]

J. Agric. Urban Entomol. 25(4): 223–232 (October 2008)

**ABSTRACT** The Argentine ant, *Linepithema humile* (Mayr), is a pest in many urban areas of the southeastern United States. In the Piedmont region of South Carolina, there is a documented problem with *L. humile* invading the campsites of state park campgrounds. While some parks have tried to implement proactive control programs, the most widely used tactic is spraying insecticides when *L. humile* populations become intolerable and visitors complain. For this study, a series of four trials were conducted to evaluate insecticidal spray treatments versus combinations of insecticidal sprays and granular bait. Control areas were set up to evaluate the change in ant foraging numbers over a season without chemical interference. One bait (Niban® Granular Bait) and three insecticidal sprays (Premise® 2, Temprid™ SC, and Tempo® Ultra SC) were evaluated to determine the best option in a park setting. The granular bait did not perform as well as the liquid insecticides. Temprid™ SC and Tempo® Ultra SC both resulted in a decrease in *L. humile* over the four-week period that was not significantly different. Although both insecticides were comparable in results, we found Tempo® Ultra SC to be the best choice in this study due to its lower cost and lower toxicity to non-target organisms. Assessing different methods of chemical management will enhance future efforts at sustainable control of *L. humile* in campground environments.

**KEY WORDS** Hymenoptera, Formicidae, *Linepithema humile*, Argentine ant, integrated pest management

*Linepithema humile* (Mayr), the Argentine ant, was probably introduced into the United States in 1891 (Newell & Barber 1913). This species can be found in states ranging from Washington, Arizona, Indiana, to Maryland, but has become an established pest in Hawaii, California and throughout the southern United States (Mallis et al. 2004). *Linepithema humile* has been reported to inhabit all continents, except Antarctica, as well as many oceanic islands (Suarez et al. 2001). In 2000, a survey of pest management professionals reported that *L. humile* ranked among the top five pest ant species in the United States (Hedges 2000). According to Aron et al. (1990), *L. humile* is ecologically successful for

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three reasons: 1) it can tolerate a variety of habitats; 2) it has polydomic and polygynic colonies; and 3) it can lay down pheromone trails.

*Linepithema humile* is an effective scavenger due to its use of mass recruitment and trail pheromones after finding a food resource (Aron et al. 1990). They can recruit cohorts up to 6 mm away from an active trail (Van Vorhis Key et al. 1981). By laying down a pheromone trail on the way to a food source and when returning to its nest, *L. humile* has an advantage over other ant species in its ability to exploit food sources at a greater rate (Aron et al. 1990, Deneubourg et al. 1990). Human & Gordon (1996) attribute the high number of *L. humile* concentrated in an area to a reduction in ant species richness, leaving *L. humile* able to dominate entire habitats (Holldobler & Wilson 1977).

Due to the recruitment behavior of *L. humile*, baiting is one viable option for control. Baits are an attractive option for treating pest ants because their use usually results in applications of smaller, concentrated amounts of insecticide (Taniguchi et al. 2005), rather than spraying a broad area with liquid toxicant. Klotz (1998) found that when low concentrations of liquid boric acid ($\leq$1%) were offered to trailing *L. humile*, there was a reduction in ants entering surrounding buildings where they previously retrieved food. In Hawaii, it was reported that, when offered either liquid sucrose baits or granular baits, *L. humile* readily accepted either formulation (Krushelnycky & Reimer 1998). Silverman & Roulston (2003) reported that whether scattered or clumped, there is no difference in the total amount of granular bait gathered.

Another strategy that takes advantage of *L. humile's* foraging behavior is the application of non-repellent chemical barriers. Rust et al. (1996) reported that barrier sprays were effective, but only within the first 30 d after application, due to uncontrollable environmental factors. Horizontal transfer of insecticide from barrier treatments was effective in killing worker ants, through both live and dead ants that had come into contact with the insecticide (Soeprono & Rust 2004). Vail & Bailey (2002) tested whether perimeter baits, sprays, or a combination were most efficient in treating the odorous house ant, *Tapinoma sessile* (Say), in residential areas. They reported that bait-only treatments did not perform as well as perimeter spray applications. Although perimeter spray-only treatments were not statistically different from the bait-spray combination treatments, a combination of both treatments was reported to be more effective for eliminating >94% of the population for up to four months.

In the Piedmont region of South Carolina, there is a documented problem with *L. humile* invading campsites in state park campgrounds. *Linepithema humile* has been reported to infest personal recreational vehicles, tents, public facilities, and a variety of locations accessed by campers (S. Hutto, SC Parks Dept., personal communication, 2006). While some state parks have attempted control, the most widely used tactic is applying liquid insecticides when *L. humile* populations become "unbearable" in certain campsites. Seasoned campers come prepared with their own "control" methods, powders and sprays that can be placed around sleeping and eating areas. Some of the methods encountered include insecticidal dusts and sprays, cleaning powders, bleach, WD-40 spray lubricant, and other home remedies passed from camper to camper (Ellis, unpublished data). When walking through campsites, it is not uncommon to see rings of insecticide or cleaning powder in many sites around the campgrounds. According to Rust et al. (2003), spray and dust applications, in which the treatment is placed around the tires of a recreational

vehicle (RV), have been used in campgrounds for years to deter the invading pests. While this does appear to repel *L. humile* from entering vehicles, the powders do not appear to be toxic (Rust et al. 2003). In a preliminary 2007 survey of three South Carolina state park campgrounds, we determined *L. humile* resides in specified locations throughout the prime camping season. Based on this information, we determined that a targeted treatment program was possible. The objective of this study was to evaluate an effective treatment strategy for *L. humile* in state park campgrounds in South Carolina.

## Methods and Materials

**Study area and treatment designation.** In the summer of 2006, ten South Carolina state parks were surveyed to determine which had high *L. humile* populations. Voucher specimens were placed in the Clemson University Arthropod collection. Three parks with the highest populations were selected for further research. In 2007, we observed that *L. humile* was not distributed throughout each park, but was instead localized around most frequently used campsites and areas in close proximity to water.

This study was conducted in Baker Creek State Park (McCormick, SC; 82°21′19.008″W, 33°53′36.996″N), Calhoun Falls State Park (Calhoun Falls, SC; 82°35′29.004″W, 34°1′23.016″N), and Lake Greenwood State Park (Ninety Six, SC; 81°58′0.8868′W, 34°11′58.7904″N) from June to September 2008. Between three and five campsites with known ant infestations in each park were selected to evaluate different chemical treatments. Each campsite was equipped for RV parking, and had water and electrical hook-up access, trees around the perimeter, and a high occupancy rate.

Three treatment areas (one control area and two treated areas) were assigned to each park. They were at least 60 m apart to prevent the interaction of ants foraging from one area to another (Vega & Rust 2003). Control areas were locations not treated with insecticides by park personnel, but still frequented by campers. Due to the actions of park visitors, each research area had the potential for other, camper-deployed chemical treatments.

**Monitoring.** Prior to insecticide application, landmarks were chosen to facilitate observation of ant numbers throughout the duration of each insecticide trial. Landmarks consisted of trees and cross-ties where heavy foraging trails were present. Once each landmark was chosen, the ant trail with the greatest number of individuals was counted for 30 s and recorded. Counts were achieved by recording the number of ants that crossed an arbitrary line, in both up and down directions (Moreno et al. 1987). All counts within a given treatment area were summed weekly and averaged to provide a mean number of ants per landmark (Rust et al. 2000). Ant trails were counted at weeks 0, 1, 2, 3, and 4.

**Treatment variations.** In Trial 1, both Treatment 1 and Treatment 2 hard-scape areas were sprayed with a 0.1% formulation of Premise® 2 (21.4%; imidacloprid; Bayer Environmental Science, 2 T.W. Alexander Drive, Research Triangle Park, NC 27709). Hard-scape was defined as all pavement edges surrounding parking areas, stairs, and crosstie bases encompassing each campsite. In addition to hard-scape applications, Treatment 1 sites had Premise® 2 applied to trees within 3 m of each campsite, according to label specification. An attempt was made to use minimal amounts of spray by targeting a limited

number of *L. humile* foraging areas, yet enough to achieve control. In addition to hard-scape spraying, treatment 2 sites also had Niban® Granular Bait (5.0% orthoboric acid; Nisus Corporation, 100 Nisus Drive, Rockford, TN 37853) placed around trees within 3 m of each campsite.

In Trial 2, the same procedures were performed as in Trial 1; however, more spray was applied to ant trails and visible nests (Table 1). Crosstie bases were treated as before, but Trial 2 consisted of additional spraying to each crosstie in a wall stack and in grooves where ants could potentially trail. All ants trailing on the ground and nests were sprayed as encountered.

In Trial 3, the same methods performed in Trial 2 were applied, but an experimental insecticide, Temprid™ SC (21.4% imidacloprid; 11.8% ß-cyfluthrin; Bayer Environmental Science), was exchanged for Premise® 2 for treatment of hard-scapes. Trees within 3 m of Treatment 1 sites were sprayed with Temprid™ SC as in previous trials, and Niban® Granular Bait was once again used around trees in Treatment 2 areas.

In Trial 4, Treatment 1 was applied in the same manner as Trial 3, again using Temprid™ SC. In Treatment 2, hard-scapes and trees within 3 m of each campsite were sprayed with a 0.025% formulation of Tempo® Ultra SC (11.8%; ß-cyfluthrin; Bayer Environmental Science) in the same manner as in Treatment 1.

For each trial, all ants counted in foraging trails within a treatment area were averaged. The objectives were to determine if there were differences among the treatments and if any of the treatments resulted in a decline in *L. humile* numbers. Ant numbers were averaged across parks to produce treatment means for each trial. The mean numbers were re-expressed as a mean change from the original number to adjust for differences among population levels of ants in the parks. The mean changes in ant numbers for each treatment were compared by Analysis of Variance (ANOVA) followed by means separation through Fisher's LSD test. The mean changes were also compared to zero using a form of Fisher's LSD test. If the mean number of ants for an individual treatment was found to be significantly less than zero, this was taken as evidence that the treatment was having some effect in reducing ant numbers. All calculations were performed using the Statistical Analysis System (SAS 2003).

## Results and Discussion

Klotz (1998) found that liquid boric acid baits were attractive and effective in decreasing *Linepithema humile* (Mayr) around structures, but in the campground setting, this is a more complex task. Due to the curiosity of wildlife and

**Table 1. Mean amount of spray insecticide used (L/campsite) in Treatment 1 and Treatment 2 areas ± standard error in Trial 1 and Trial 2.**

| | Treatment 1 (L/campsite) | Treatment 2 (L/campsite) |
|---|---|---|
| Trial 1 | 2.84 ± 0.29 | 2.15 ± 0.30 |
| Trial 2 | 4.66 ± 0.54 | 2.43 ± 0.42 |

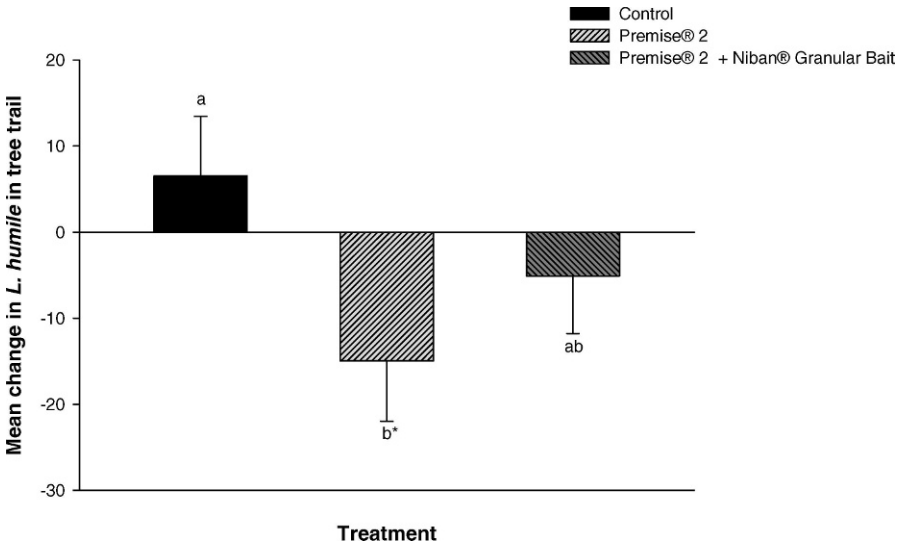


**Fig. 1.** Combined mean change in number of *Linepithema humile* (Mayr) present in foraging trails at Baker Creek, Calhoun Falls, and Lake Greenwood State Parks over the course of Trial 1. Different letters represent a significant difference at $\alpha = 0.05$, and * indicates significance less than 0 (means were compared using ANOVA followed by Fisher's LSD).

park visitors, it is necessary to use containers that discourage tampering or consumption of bait product. Appropriate containers need to provide adequate amounts of bait to sate the high populations of *L. humile* recruited to the food source. Such containers also need to inhibit microbial growth and reduce evaporation, as well as protect the bait from an influx of water (precipitation or irrigation), to keep it from becoming unpalatable, and thus ineffective (Silverman & Brightwell 2008). Finding a station design that would satisfy these requirements, yet allow *L. humile* to actively forage upon the enclosed bait, was a problem. Previous attempts have been made in campgrounds with makeshift stations containing Terro® Liquid Ant Bait (Senoret Chemical Co., Inc., 566 Leffingwell Ave., St. Louis, MO, 63122), but depleted containers were rarely refilled and thus remained empty and ineffective (S. Hutto, SC Parks Dept., personal communication, 2006). However, when offered either liquid sucrose baits or granular baits, *L. humile* readily accepted either formulation (Krushelnycky & Reimer 1998). During a preliminary survey of several granular baits in May 2008, we determined that *L. humile* recruited mostly to a boric-acid based granular formulation. For this reason Niban® Granular Bait was selected for our trials.

The combined mean change of *L. humile* in foraging trails observed over the four-week Trial 1 period (Fig. 1) indicated a significant difference between the control area and the areas treated only with Premise® 2 (Treatment 1) ($t = -2.86$, df = 4, $P = 0.0461$). However, no significant differences were found between the control and Premise® 2 + Niban® Granular Bait treated areas (Treatment 2) ($t = -1.61$, df = 4, $P = 0.1825$) or between Premise® 2-only and Premise® 2 + Niban®

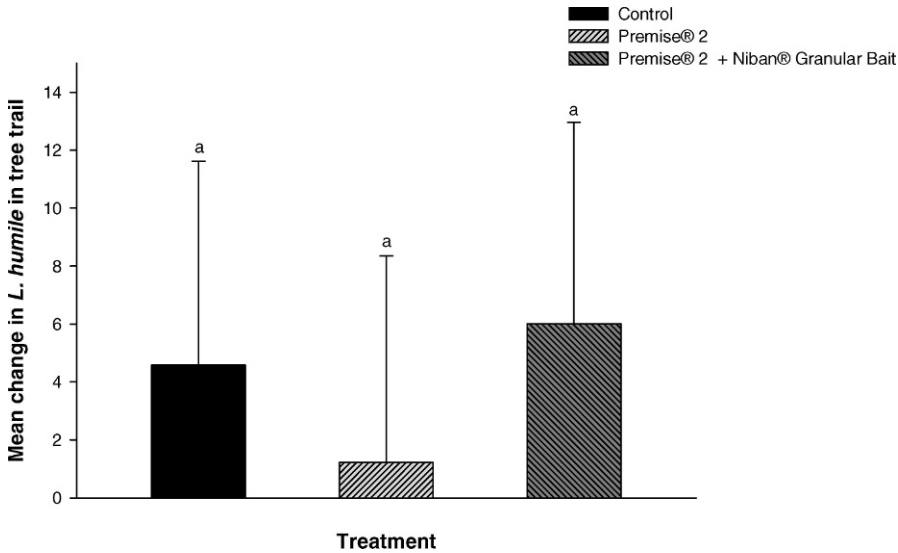


**Fig. 2.** Combined mean change in number of *Linepithema humile* (Mayr) present in foraging trails at Baker Creek, Calhoun Falls, and Lake Greenwood State Parks over the course of Trial 2. Different letters represent a significant difference at $\alpha = 0.05$ (least squares means test).

Granular Bait treated areas ($t = -1.36$, df = 4, $P = 0.2447$) (Fig. 1). Although there was no significant difference between Premise® 2-only treated areas and Premise® 2 + Niban® Granular Bait treated areas, there was a larger decrease in the mean number of ants trailing over the four-week period in Premise® 2-only treated areas that indicated a significance of less than 0 ($t = -2.13$, df = 4, $P = 0.04995$). Because treatments were not significantly different, we decided the trial should be repeated. The second trial included more attentive spraying of surrounding hard-scape areas as well as directed spraying of trailing ants and visible nests. In Trial 2, even though spraying was more thorough, both treatment and control areas were not statistically different, and *L. humile* increased over the four week period in all areas (Fig. 2).

In Trial 3, the insecticide was changed to Temprid™ SC, which is formulated with active ingredients from both Premise® 2 (imidacloprid) and Tempo® Ultra SC (ß–cyfluthrin). We observed no significant difference between the control and Temprid™ SC + Niban® Granular Bait treated areas (Treatment 2) ($t = -1.17$, df = 4, $P = 0.3060$) (Fig. 3). However, a significant difference was found between the control and Temprid™ SC-only treated areas (Treatment 1) ($t = -3.95$, df = 4, $P = 0.0168$), as well as between Temprid™ SC-only and Temprid™ SC + Niban® Granular Bait treated areas (Treatment 2) ($t = -2.86$, df = 4, $P = 0.0457$). The control and Temprid™ SC + Niban® Granular Bait treated areas showed an increase in *L. humile*, while Temprid™ SC-only areas indicated a decrease in the mean number of trailing *L. humile* over the four week period ($t = -2.81$, df = 4, $P = 0.02425$) (Fig. 3).

During Trial 3, we found that Temprid™ SC-only was more effective than Temprid™ SC + Niban® Granular Bait. Because granular bait had not been as

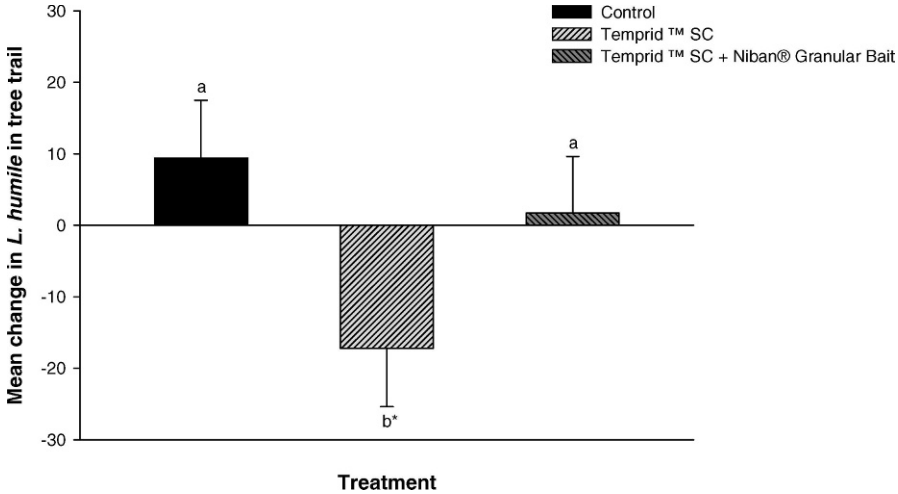


**Fig. 3.** Combined mean change in number of *Linepithema humile* (Mayr) present in foraging trails at Baker Creek, Calhoun Falls, and Lake Greenwood State Parks over the course of Trial 3. Different letters represent a significant difference at $\alpha = 0.05$, and * indicates significance less than 0 (means were compared using ANOVA followed by Fisher's LSD).

effective as liquid insecticide in reducing ant numbers during the first three trials, during Trial 4 only liquid insecticides was chosen for treatment in both areas. Again, Temprid™ SC (imidacloprid and ß-cyfluthrin) was used as Treatment 1, but Tempo® Ultra SC (ß-cyfluthrin) was used as Treatment 2. Tempo® Ultra SC was selected because Premise® 2 (imidacloprid) had already been tested alone, and we questioned if it was a particular component of the Temprid™ SC causing the effectiveness or the combination of the two active ingredients. The mean change of *L. humile* in foraging trails observed over the four week period of Trial 4 indicated a significant difference between the control and Tempo® Ultra SC treated areas (Treatment 2) ($t = -3.49$, df = 4, $P = 0.0252$) (Fig. 4). However, no significant difference was found between the control and Temprid™ SC treated areas (Treatment 1) ($t = -0.95$, df = 4, $P = 0.3943$). We found both Temprid™ SC-only and Tempo® Ultra SC-only treated areas were statistically not different ($t = 2.50$, df = 4, $P = 0.0667$). However, there was a greater decrease in *L. humile* numbers in Tempo® Ultra SC-only treated areas indicating a significance less than 0 ($t = -3.19$, df = 4, $P = 0.0288$).

If selecting a treatment for *L. humile* in park campgrounds were based only on efficacy, Temprid™ SC-only or Tempo® Ultra SC-only treatments would be the treatments of choice. However, in a campground setting, product cost to control a pest is important. In our trials, the treatment cost for Tempo® Ultra SC was \$0.37/L and for Temprid™ SC it was \$0.86/L; thus, Tempo® Ultra SC was the more cost-effective choice. When treating a sensitive environment where water, wildlife, and humans can be affected, many factors beyond price must be considered. If using the lowest amount of active ingredient in the campground is the prime consideration, Tempo® Ultra SC also is preferred. Temprid™ SC was

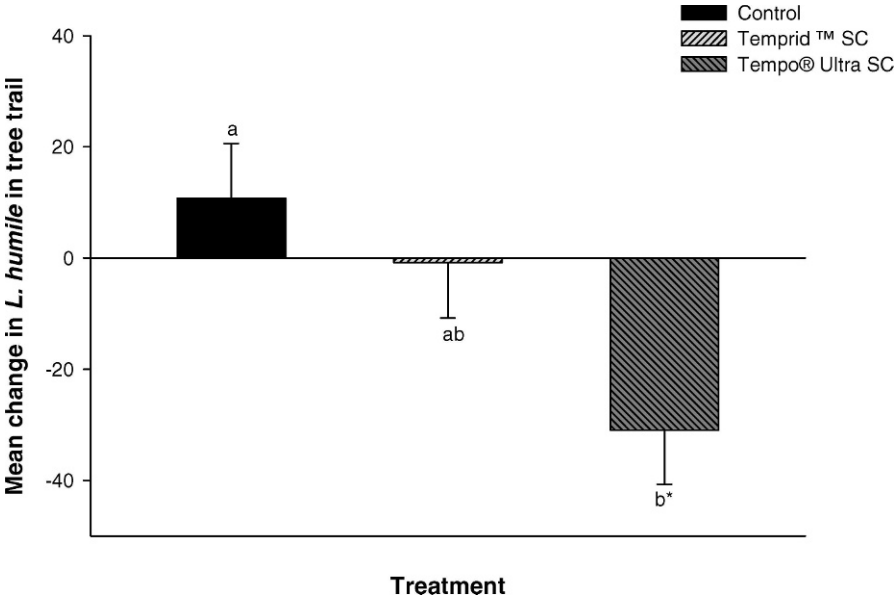


**Fig. 4.** Combined mean change in number of *Linepithema humile* (Mayr) present in foraging trails at Baker Creek, Calhoun Falls, and Lake Greenwood State Parks over the course of Trial 4. Different letters represent a significant difference at $\alpha = 0.05$, and * indicates significance less than 0 (means were compared using ANOVA followed by Fisher's LSD).

applied at 0.25 ml/L and 0.45 ml/L A.I. (ß-cyfluthrin and imidacloprid, respectively), but Tempo® Ultra SC was applied at a rate of only 0.25 ml/L (imidacloprid). Not only did Tempo® Ultra SC treatments result in less active ingredient being applied than in Temprid™ SC treatments, but also the active ingredient of Tempo® Ultra SC has a lower toxicity to non-target organisms.

When deciding on the best option for treating an area, two strategies can be used: 1) one to three larger volume insecticide applications or 2) many smaller volume applications. Many smaller volume applications could cost more over time due to product usage and cost of applicator time, potentially place more insecticide into the environment, and possibly magnify the current pest problem (Silverman & Brightwell 2008). Although an alternate to liquid insecticide applications for *L. humile* management at SC state parks is desirable, the use of targeted Tempo® Ultra SC treatments results in less insecticide being applied compared to the current park program. The current practice is to spray an area if campers complain, even if this means spraying sites more than once a week. The single, larger volume spray per trial used in this research project was chosen in an attempt to reduce the amount of insecticide entering the campgrounds over the season.

Although the use of boric acid granular bait was discontinued after Trial 3, further research to test bait efficacy against *L. humile* in the campgrounds earlier in the year may be useful. Because our trials did not begin until June, there may be several reasons why targeted baiting performed poorly. In June, *L. humile*

populations were peaking and the high number of foraging ants may have overwhelmed the available bait. Brood production was declining and workers may not have been foraging for the nutritional components offered in the bait selected. Finally, food sources from campers and honeydew-secreting insects in the area may have competed with the bait.

In conclusion, it was found that the best choice for reducing *L. humile* in this study was Tempo® Ultra SC from June-August in a spray application to hardscapes, visible trails and nests, and the bases of trees within 3 m of each campsite. However, there may be application strategies and combinations of products that could be more effective at different times of the year, including, perhaps, targeted treatments with scatter baits applied earlier in the season.

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# Evaluation of Chemical Control Strategies for *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) in South Carolina State Park Campgrounds[1]

Brittany R. Ellis,[2] Eric P. Benson,[2] Patricia A. Zungoli,[2] and William C. Bridges, Jr.[3]

J. Agric. Urban Entomol. 25(4): 223–232 (October 2008)

**ABSTRACT** The Argentine ant, *Linepithema humile* (Mayr), is a pest in many urban areas of the southeastern United States. In the Piedmont region of South Carolina, there is a documented problem with *L. humile* invading the campsites of state park campgrounds. While some parks have tried to implement proactive control programs, the most widely used tactic is spraying insecticides when *L. humile* populations become intolerable and visitors complain. For this study, a series of four trials were conducted to evaluate insecticidal spray treatments versus combinations of insecticidal sprays and granular bait. Control areas were set up to evaluate the change in ant foraging numbers over a season without chemical interference. One bait (Niban® Granular Bait) and three insecticidal sprays (Premise® 2, Temprid™ SC, and Tempo® Ultra SC) were evaluated to determine the best option in a park setting. The granular bait did not perform as well as the liquid insecticides. Temprid™ SC and Tempo® Ultra SC both resulted in a decrease in *L. humile* over the four-week period that was not significantly different. Although both insecticides were comparable in results, we found Tempo® Ultra SC to be the best choice in this study due to its lower cost and lower toxicity to non-target organisms. Assessing different methods of chemical management will enhance future efforts at sustainable control of *L. humile* in campground environments.

**KEY WORDS** Hymenoptera, Formicidae, *Linepithema humile*, Argentine ant, integrated pest management

*Linepithema humile* (Mayr), the Argentine ant, was probably introduced into the United States in 1891 (Newell & Barber 1913). This species can be found in states ranging from Washington, Arizona, Indiana, to Maryland, but has become an established pest in Hawaii, California and throughout the southern United States (Mallis et al. 2004). *Linepithema humile* has been reported to inhabit all continents, except Antarctica, as well as many oceanic islands (Suarez et al. 2001). In 2000, a survey of pest management professionals reported that *L. humile* ranked among the top five pest ant species in the United States (Hedges 2000). According to Aron et al. (1990), *L. humile* is ecologically successful for

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# Relative Efficacies of Congo Red and Tinopal LPW on the Activity of the Gypsy Moth (Lepidoptera: Lymantriidae), Nucleopolyhedrovirus and Cypovirus[1]

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**ABSTRACT** Congo red (a diazo dye) and Tinopal LPW (a stilbene fluorescent brightener) were assayed to determine their effects on the activity of the gypsy moth, *Lymantria dispar* (L.), nucleopolyhedrovirus (LdMNPV) and cypovirus (LdCPV) infecting the gypsy moth. In the case of LdMNPV, Congo red reduced the median lethal concentration ($LC_{50}$) by approximately 26-fold, whereas Tinopal LPW reduced the $LC_{50}$ by approximately 360-fold. In tests using late second-stage larvae treated with LdCPV, $LC_{50}$s were reduced by approximately 12-fold and approximately 430-fold by Congo red and Tinopal LPW, respectively. Fourth instar larvae were challenged with LdMNPV/water, LdMNPV/Congo red, and LdMNPV/Tinopal LPW and the activities of progeny virus from all treatments were similar.

**KEY WORDS** *Lymantria dispar*, gypsy moth, nucleopolyhedrovirus, cypovirus, Congo red, Tinopal LPW

Insect pathogenic viruses are logical candidates for use in pest management systems, because they are active against many agriculturally important lepidopteran pests (Ignoffo 1975, Moscardi 1999, Szewczyk et al. 2006). Their use, however, has been limited by such factors as slow speed of kill and sensitivity to sunlight (Dougherty et al. 1996, Arivudainambi et al. 2000, Inceoglu et al. 2006). During the course of research on ultraviolet (UV) protectants, such chemicals as Congo red (a diazo dye) and stilbene optical brighteners like Phorwite AR, Leucophor BS, Leucophor BSB (Shapiro 1992) were found to provide excellent UV protection. The addition of Congo red and stilbene brighteners such as Phorwite AR to the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), nucleopolyhedrovirus (LdMNPV) resulted in faster kill and lower $LC_{50}$s (Shapiro 1989, Shapiro & Robertson 1992, Shapiro et al. 1992). A subsequent study indicated that Tinopal LPW enhanced the activity of a cypovirus LdCPV against gypsy moth larvae (Sheppard & Shapiro 1994, Sheppard et al. 1994).

Since the discovery that stilbene optical brighteners could act as virus enhancers (Shapiro & Robertson 1992, Shapiro et al. 1992), many studies have been conducted that confirmed and expanded our knowledge of the spectrum of brightener activity both in the laboratory and in the field (Moscardi 1999, El

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Salamouny 2007, Wang et al. 2007a). Studies devoted to Congo red and insect pathogens demonstrated its effectiveness as a UV protectant (Shapiro 1989, Ignoffo et al. 1991, Ramirez-Lepe et al. 2003). Although Congo red and Tinopal LPW are chemically different, both form hydrogen bonds with various polysaccharides (Maeda & Ishida 1967, Kopecka & Gabriel 1992) and have affinity for cellulose and chitin (Haigler et al. 1980, Herth 1980). Moreover, both chemicals have been shown to inhibit chitin synthesis and/or assembly (Herth 1980, Bartnicki-Garcia et al. 1994, Garcia-Zapien et al. 1999). Because these chemicals may possess similar biological activities, we conducted a study to investigate the effects of Congo red on the susceptibility of gypsy moth larvae to a baculovirus (LdMNPV) and a cypovirus (LdCPV) and to determine whether the virulence of progeny virus from LdMNPV/Congo red treatment and progeny virus from LdMNPV/Tinopal LPW treatment was changed after serial passage through *L. dispar* larvae.

## Materials and Methods

**Insects, Virus Inocula.** The colonized New Jersey strain of the gypsy moth (established and maintained by USDA-APHIS, Otis ANGB, MA) was used. The gypsy moths were reared on a wheat germ diet (Bell et al. 1981). LdCPV was obtained from the Asian Parasite Research Laboratory (USDA, Seoul, Korea) and was subsequently passed through gypsy moth larvae (Shapiro et al. 1994). The LdMNPV isolate LDP-226 was obtained as a freeze-dried powder from the U. S. Forest Service, Hamden, CT and has been used as a virus standard.

**Test Chemicals.** Congo red (CR) (= 3,3′-1,1′biphenyl-4-4-diylbis(azo)bis[4-amino-1-naphthalene sulfonic acid] disodium salt; Direct red; CAS # 573-58-0) and Tinopal LPW (= disodium,4,4′-bis[(4-anilo-6-morpholino-1,3,5-triazin-2-yl)-amino]stilbene-2,2′-disulfonate; Calcofluor M2R; Fluorescent brightener 28; CAS # 4404-43-07) were obtained as technical powders (Sigma-Aldrich, St. Louis, MO) and were diluted in distilled water.

**Congo red, Bioassays.** LdMNPV [= $4.35 \times 10^{10}$ virus occlusion bodies (OBs) per gram] was initially diluted in distilled water to obtain a concentration of $10^7$ OB/ml. Subsequently, LdMNPV was diluted in distilled water (virus standard) or in aqueous solutions of Congo red to produce concentrations of $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ OBs/ml (=$10^{-1}$ to $10^3$ OBs per mm$^2$ of diet surface). One ml of each virus suspension was pipetted onto the surface of each 180-ml container (=4,770 mm$^2$) (W. L. Enterprises, Newark, NJ) containing 10 second-stage larvae (7-d-old; mean ± SE weight, 35 ± 6.8 mg per larva). Tests were repeated six times. In all tests, 30 larvae per virus dilution per treatment per replicate, 30 untreated larvae per replicate, and 30 Congo red-treated larvae per replicate were used. Larvae were maintained at 29°C, 50% RH and a 12:12 (light:dark) photoperiod and mortality readings were recorded on day 7 and every other day thereafter until day 21.

**Congo red, Tinopal LPW, Bioassays.** In subsequent tests, Congo red (0.014 M) and Tinopal LPW (0.010 M) were compared as enhancers for LdMNPV and LdCPV, using the same protocol. Viruses were applied to the diet surface at concentrations ranging from $2 \times 10^{-3}$ to $2.1 \times 10^2$ OB/mm$^2$ of diet surface. Tests were repeated five times.

**Congo red, Tinopal LPW, Virus production, Progeny Virus.** To determine the effects of Congo red and Tinopal LPW on LdMNPV activity, virus replication, and progeny virus, late fourth instars were used as test insects. One thousand five hundred neonates were placed on diet (10 per 180-ml cup) and were reared under standard conditions. Late fourth instars were randomly selected and 400 larvae per treatment were challenged with LdMNPV/$H_2O$ (virus standard), LdMNPV/CR (0.014M), or LdMNPV/LPW (0.014M) at a virus concentration of $4.77 \times 10^2$ OB/mm$^2$ (= $LC_{90}$ for LdMNPV). In addition, 100 untreated controls, 100 CR-treated larvae, and 100 LPW-treated larvae were tested. At days 7, 11, and 15, mortality readings were recorded and 100 larvae from each virus-treated group were removed at each time period and stored at $-20°C$.

Virus was harvested separately from each group of frozen larvae (Shapiro et al. 1981) and virus yields (= OBs per larva) were determined. Biological activities of the progeny virus obtained from each test group also were determined. Occlusion bodies from each group were counted five times to obtain an average. Each sample was diluted in distilled water and applied to the diet surface (1 ml/180-ml cup) at concentrations ranging from $2 \times 10^{-2}$ to $2.1 \times 10^2$ OB/mm$^2$ of diet surface. Tests were repeated five times with 30 larvae per virus dilution per treatment, 30 untreated control larvae, 30 CR-treated larvae, and 30 LPW-treated larvae per replicate. Mortality was initially assessed at day 7 and was recorded every other day until day 21.

**Statistical Methods.** Concentration-mortality regressions were calculated to determine the effects of Congo red and Tinopal LPW on virus activities. Slopes and $LC_{50}$s were obtained with the probit option of POLO (LeOra Software, 1987). After ANOVA, means were separated for significance according to Fisher's Protected Least Significant Difference (LSD) test. Each treatment was compared to the standard (= LdMNPV/$H_2O$, 0 UV) as well as to each other according to Student's 2-tailed *t*-test (Steel & Torrie 1980). A statistical program was used to determine the relationship (correlation coefficient) between Congo red concentration and enhancement activity (Easy Calculation.com 2009).

## Results

**Congo red, LdMNPV.** The enhancement activity of Congo red was concentration-dependent ($r^2 = 0.94$) (Table 1). Congo red at 0.0014 M had little effect upon LdMNPV activity, but an increase in concentration to 0.0035 M reduced the $LC_{50}$ value by 2.3-fold. As the Congo red concentration increased to 0.0070M, the $LC_{50}$ further decreased by another 1.9-fold. The greatest increase in activity was observed at the 0.0140 M level (a 4.9-fold increase from the 0.0070 M Congo red treatment), and the $LC_{50}$ value for the LdMNPV/Congo red (0.140 M) treatment was 20.7-fold lower than the $LC_{50}$ value for the LdMNPV/$H_2O$ (standard) treatment (Table 1). No larval mortality occurred among any Congo red-treated larvae.

When fourth-stage larvae were exposed to a single concentration of LdMNPV (= $4.77 \times 10^2$ OB/mm$^2$; = $LC_{90-95}$), no larvae died during the first 7 days. By day 11, approximately 40% of the larvae died from virus, and by day 15, approximately 95% of the larvae died. The addition of Congo red (0.014 M) to LdMNPV also resulted in greater initial viral occlusion body yields among

**Table 1. $LC_{50}$ (95% CL) and Activity Ratio (AR) of the gypsy moth nucleopolyhedrovirus LdMNPV with and without the addition of Congo red: Effect of concentration.**

| Congo red treatment LdMNPV + (M) | $LC_{50}$ (95% CL)[a] | AR[b] | Slope (± SE) |
|---|---|---|---|
| $H_2O$ (Control) | 6.20 (5.61–6.80)[a] | 1.00 | 2.21 (0.02) |
| 0.0014 | 6.45 (5.65–7.26)[a] | 0.96 | 1.97 (0.02) |
| 0.0035 | 2.77 (1.98–3.56)[b] | 2.24 | 1.60 (0.01) |
| 0.0070 | 1.46 (0.61–2.30)[c] | 4.25 | 1.57 (0.01) |
| 0.0140 | 0.30 (0.20–0.40)[d] | 20.67 | 1.53 (0.01) |

[a]$LC_{50}$s are expressed as OBs per $mm^2$ of diet surface; six replicates; five virus concentrations per virus treatment per replicate; 30 untreated larvae per replicate; 30 Congo-red treated larvae per concentration per replicate.
[b]Activity Ratio (AR) is calculated by dividing the $LC_{50}$ for LdMNPV/$H_2O$ (=virus standard) by the $LC_{50}$ for LdMNPV/Congo red. The AR value for the virus standard = 1.00.
Means followed by different letters within a column were significantly different (Fisher's Protected Least Significant Difference (LSD); $\alpha = \leq 0.05$).

mature larvae. The OB yields per larva at day 7 were approximately 2-fold greater (i.e., $1.2 \times 10^9 \pm 0.2 \times 10^9$) among those larvae exposed to LdMNPV/Congo red than among those exposed to LdMNPV/$H_2O$ ($7.0 \times 10^8 \pm 0.1 \times 10^8$). At day 11, however, yields from LdMNPV/$H_2O$-treated larvae increased to $2.6 \times 10^9 \pm 0.4 \times 10^9$ OBs per larva and were approximately 2-fold greater than those obtained for LdMNPV/Congo red-treated larvae ($1.3 \times 10^9 \pm 0.2 \times 10^9$). By day 14, OB yield remained unchanged among LdMNPV/$H_2O$-treated larvae ($2.6 \times 10^9 \pm 0.3 \times 10^9$ OBs) and the virus yield increased from 1.3 to $1.7 \times 10^9$ ($\pm 0.1 \times 10^9$) OBs per LdMNPV/Congo red-treated larvae.

**Congo red, Tinopal LPW, LdMNPV, LdCPV.** Congo red (0.014M) and Tinopal LPW (0.010 M) were compared as synergists for two different viruses of the gypsy moth (i. e., the nucleopolyhedrovirus LdMNPV and the cypovirus LdCPV) in bioassays with second instars The addition of Congo red to LdMNPV and LdCPV reduced $LC_{50}$s by approximately 26-fold (Table 2) and 12-fold, respectively (Tables 2, 3), whereas the addition of Tinopal LPW to LdMNPV and LdCPV reduced $LC_{50}$s by approximately 382-fold and 430-fold, respectively (Tables 2, 3). When late fourth instars were challenged and progeny virus was collected from virus-killed larvae, the activities of progeny virus from the LdMNPV/Congo red and the LdMNPV/Tinopal LPW treatments were similar, regardless of the original treatment. $LC_{50}$s for these virus isolates were 8.26 OBs/$mm^2$ (5.35–12.86, 95% CL; slope = 1.76 ± 0.04) for LdMNPV/$H_20$, 8.18 OBs/$mm^2$ (5.62–11.93 95% CL; slope = 1.87 ± 0.04) for LdMNPV/Congo red, and 7.71 OBs/$mm^2$ (5.05–11.80, 95% CL; slope = 1.79 ± 0.04) for LdMNPV/Tinopal LPW progeny virus.

## Discussion

The peritrophic membrane, comprised of protein, chitin, and mucin (Lehane 1997, Wang & Granados 2000), may serve as an effective barrier for the invasion

**Table 2. $LC_{50}$ and Activity Ratio (AR) of the gypsy moth nucleopolyhedrovirus LdMNPV with and without Congo red and Tinopal LPW.**

| Treatment | $LC_{50}$ (95% CL)[a] | AR[b] | Slope (± SE) |
|---|---|---|---|
| LdMNPV/$H_2O$ | 7.63 (5.28–11.01)[a] | 1.00 | 2.25 (± 0.03) |
| LdMNPV/Congo red[c] | 0.29 (0.14–0.67)[b] | 26.31 | 1.84 (± 0.04) |
| LdMNPV/Tinopal LPW[d] | 0.02 (0.01–0.03)[c] | 381.50 | 1.96 (± 0.02) |

[a]$LC_{50}$ values are expressed as OBs per $mm^2$ of diet surface; five replicates; five virus concentrations per virus treatment per replicate; 30 larvae per virus concentration per treatment per replicate; 30 untreated control per replicate; 30 Congo red-treated and 30 Tinopal LPW-treated larvae per replicate.
[b]Activity Ratio (AR) is calculated by dividing the $LC_{50}$ for LdMNPV/$H_2O$ (= virus standard) by the $LC_{50}$ for LdMNPV/test chemical.
[c]Congo red was used at a final concentration of 0.014 M.
[d]Tinopal LPW was used at a final concentration of 0.010 M.
Means followed by different letters within the same column were significantly different [Fisher's Protected Least Significant Difference (LSD)]; $\alpha = \leq 0.05$).

of microorganisms, including insect viruses (Brandt et al. 1978, Wang & Granados 1998, Levy et al. 2007). Conversely, the disruption of the chitin (Shapiro et al. 1987, Shternshis et al. 2002) or mucin (Wang & Granados 1997, Peng et al. 1999) components of the peritrophic membrane results in increased mortality due to insect pathogenic bacteria (Smirnoff & Valera 1972, Dubois 1977, Shternshis et al. 2002), fungi (Fang et al. 2005, Fan et al. 2007), viruses (Tanada 1985, Peng et al. 1999, Zhu et al. 2007), polyoxin fungicides (Cohen 2005), benzoylaryl insecticides (Denneulin & Lamy 1977, Rojas & Morales-Ramos 2001, Guo et al. 2007), and chitinase (Dubois 1977, Shapiro et al. 1987).

**Table 3. $LC_{50}$ and Activity Ratio (AR) of the gypsy moth cypovirus with and without Congo red and Tinopal LPW.**

| Treatment | $LC_{50}$ (95% CL)[a] | AR[b] | Slope (± SE) |
|---|---|---|---|
| LdCPV/$H_2O$ | 21.49 (16.23–27.97)[a] | 1.00 | 2.25 (± 0.03) |
| LdCPV/Congo red[c] | 1.75 (0.74–2.35)[b] | 12.28 | 1.87 (± 0.02) |
| LdCPV/Tinopal LPW[d] | 0.05 (0.04–0.07)[c] | 429.80 | 1.94 (0.02) |

[a]$LC_{50}$ values were expressed as OBs/$mm^2$ of diet surface; five replicates; five virus concentrations per treatment per replicate; 30 larvae per virus concentration per treatment per replicate; 30 untreated control per replicate; 30 Congo red-treated and 30 Tinopal LPW-treated larvae per replicate.
[b]Activity Ratio (AR) was calculated by dividing the $LC_{50}$ for LdCPV/$H_2O$ (= virus standard) by the $LC_{50}$ for LdCPV/test chemical.
[c]Congo red was used at a final concentration of 0.014 M.
[d]Tinopal LPW was used at a final concentration of 0.010 M.
Means followed by different letters within the same column were significantly different [Fisher's Protected Least Significant Difference (LSD)]; $\alpha = \leq 0.05$).

For more than 30 years, scientists recognized that Calcofluor and Congo red had similar inhibitory effects on chitin and cellulose synthesis, assembly, and polymerization (Cabib & Bowers 1975, Herth 1980, Ram & Klis 2006). Chitin synthase activity is inhibited (Elorza et al. 1983, Delom et al. 2006) or not inhibited (Raclavsky et al. 1999) depending on the system used and on the concentration of Calcofluor M2R (Roncero & Durán 1985, Roncero et al. 1988). Moreover, Calcofluor M2R was shown to inhibit peritrophic membrane formation in larvae of *L. dispar*, *Pseudaletia unipuncta* Haworth (Lepidoptera: Noctuidae), *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) (Wang & Granados 2000), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Bolognesi et al. 2001), and *S. exigua* (Hübner) (Lepidoptera: Noctuidae) (Zhu et al. 2007).

We observed that both Congo red-treated insects (Shapiro 1989) and Tinopal LPW-treated insects (Sheppard & Shapiro 1994, Dougherty et al. 1996) died more quickly than those larvae exposed to LdMNPV alone. At that time however, no comparison was made between Calcofluor M2R (= Tinopal LPW) and Congo red.

In the present study, the addition of Tinopal LPW and Congo red to LdMNPV resulted in greater initial mortality (at day 7) and greater initial OB production than in LdMNPV-treated larvae (Shapiro & Robertson 1992). For LdMNPV/Tinopal LPW-treated larvae, "perhaps greater number of virus nucleocapsids (released from viral occlusion bodies by alkaline digestion in the gypsy moth foregut-midgut) penetrate the damaged peritrophic membrane, pass from the gut lumen into the hemocoel, and infect susceptible cells" (Shapiro & Robertson 1992), including normally refractive midgut epithelial cells (Shields 1985, Adams et al. 1994). Because previous studies indicated that Congo red was mutagenic (Tanaka 1980, Mathur et al. 2005, Wang et al. 2007b), weakly mutagenic (Prival & Mitchell 1982), or non-mutagenic (Arciola et al. 2001), we tested whether Congo red or Tinopal LPW treatments had any effect on the virulence of progeny virus. Reichelderfer & Benton (1973) reported that treatment with a known mutagen, 3-methylcholanthrene, resulted in an increase in virulence of the NPV from fall armyworm, *Spodoptera frugiperda*, SfMNPV. Similar results were observed with 1,2,3,4-dibenzanthrace,5-bromodeoxy- and 5-iododeoxy-uridine for the NPV from the alfalfa looper, *Autographa californica* (Speyer) (Lepidoptera: Noctuidae), AcMNPV, against the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae). However, no significant changes in virulence were observed with 5-bromodeoxyuridine or n-methyl-n′-nitrosoguanidine against *T. ni, Helicoverpa zea*, *Heliothis virescens*, or *S. frugiperda* (McClintock & Reichelderfer 1985). Wood et al. (1981) reported that treatment with 2-aminopurine resulted in a mutant with increased virulence.

When progeny LdMNPV was recovered from LdMNPV/Congo red-treated and LdMNPV/Tinopal LPW-treated larvae, no differences were observed between $LC_{50}$s for parental NPV and progeny NPV. Moreover, these results suggested that the effects of Congo red and Tinopal LPW were limited to the original passage and did not alter virulence in a subsequent passage. The results from the LdMNPV/LPW treatment were not surprising, because several stilbene fluorescent brighteners were not mutagenic in the Ames test and were negative for chromosome aberration and sister chromatid exchanges in Chinese hamster ovary cells (Center for Research Information, Inc. 2004, ETAD Fluorescent Whitening Agent Task Force 2005). Moreover, Tinopal LPW did not alter viral

protein or LdMNPV virions (Dougherty et al. 1996), did not "alter the genotypic composition of viral progeny during four successive passages of the virus" (Murillo et al. 2003), and did not affect covert NPV infection in *S. frugiperda* larvae (Martinez et al. 2005). At this time, however, the genotypic composition of progeny virus from LdMNPV-Congo red treatment is unknown.

While much of this study was centered on LdMNPV, we also investigated the effects of Congo red and Tinopal LPW as synergists for a cypovirus, LdCPV. Previously, we showed that LdCPV was significantly less active than LdMNPV against the gypsy moth and that Tinopal LPW acted as a synergist (Shapiro & Dougherty 1994, Shapiro et al. 1994). Although Congo red acted as a synergist, Tinopal LPW was 35-fold more effective than Congo red in decreasing $LC_{50}$s (Congo red increased activity by 12.3-fold and Tinopal LPW increased activity by 430-fold) (Table 3). In the case of LdMNPV, Tinopal LPW was 15-fold more effective than Congo red in lowering $LC_{50}$s (Table 2). Whereas Tinopal LPW inhibits apoptosis (Dougherty et al. 2006), inhibits midgut sloughing of infected cells (Washburn et al. 1998), increases viral replication in midgut epithelial cells (Adams et al. 1994) and acts as a virus synergist in different lepidopteran-virus systems (Li & Otvos 1999, Mukawa et al. 2003, Zhu et al. 2007), we have no information on Congo red. Moreover , we do not know the mode of action of Congo red or why Tinopal LPW is more effective than Congo red as a virus synergist for both LdMNPV and LdCPV (Tables 2, 3). However, we can conclude that dissimilar chemicals with similar biological activities (i.e., inhibition of chitin assembly or synthesis) (Arakawa 2002, 2003, Arakawa & Nozawa 2005) can act as effective synergists for insect pathogens in the control of insect pest populations (Kramer & Muthukishnan 1997).

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# Relative Efficacies of Congo Red and Tinopal LPW on the Activity of the Gypsy Moth (Lepidoptera: Lymantriidae), Nucleopolyhedrovirus and Cypovirus[1]

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**ABSTRACT** Congo red (a diazo dye) and Tinopal LPW (a stilbene fluorescent brightener) were assayed to determine their effects on the activity of the gypsy moth, *Lymantria dispar* (L.), nucleopolyhedrovirus (LdMNPV) and cypovirus (LdCPV) infecting the gypsy moth. In the case of LdMNPV, Congo red reduced the median lethal concentration ($LC_{50}$) by approximately 26-fold, whereas Tinopal LPW reduced the $LC_{50}$ by approximately 360-fold. In tests using late second-stage larvae treated with LdCPV, $LC_{50}$s were reduced by approximately 12-fold and approximately 430-fold by Congo red and Tinopal LPW, respectively. Fourth instar larvae were challenged with LdMNPV/water, LdMNPV/Congo red, and LdMNPV/Tinopal LPW and the activities of progeny virus from all treatments were similar.

**KEY WORDS** *Lymantria dispar*, gypsy moth, nucleopolyhedrovirus, cypovirus, Congo red, Tinopal LPW

Insect pathogenic viruses are logical candidates for use in pest management systems, because they are active against many agriculturally important lepidopteran pests (Ignoffo 1975, Moscardi 1999, Szewczyk et al. 2006). Their use, however, has been limited by such factors as slow speed of kill and sensitivity to sunlight (Dougherty et al. 1996, Arivudainambi et al. 2000, Inceoglu et al. 2006). During the course of research on ultraviolet (UV) protectants, such chemicals as Congo red (a diazo dye) and stilbene optical brighteners like Phorwite AR, Leucophor BS, Leucophor BSB (Shapiro 1992) were found to provide excellent UV protection. The addition of Congo red and stilbene brighteners such as Phorwite AR to the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), nucleopolyhedrovirus (LdMNPV) resulted in faster kill and lower $LC_{50}$s (Shapiro 1989, Shapiro & Robertson 1992, Shapiro et al. 1992). A subsequent study indicated that Tinopal LPW enhanced the activity of a cypovirus LdCPV against gypsy moth larvae (Sheppard & Shapiro 1994, Sheppard et al. 1994).

Since the discovery that stilbene optical brighteners could act as virus enhancers (Shapiro & Robertson 1992, Shapiro et al. 1992), many studies have been conducted that confirmed and expanded our knowledge of the spectrum of brightener activity both in the laboratory and in the field (Moscardi 1999, El

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# Resistance in Cultivated Sunflower to the Sunflower Moth (Lepidoptera: Pyralidae)[1]

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**ABSTRACT** A five-year field study evaluated 42 sunflower (*Helianthus annuus* L.) accessions, 25 breeding lines, and 40 interspecific crosses for resistance to infestation and damage from larval feeding by naturally occurring populations of the sunflower moth, *Homoeosoma electellum* (Hulst) (Lepidoptera: Pyralidae). Accessions PI 175728 and PI 307946 had less than 3% feeding damage per head in all three years they were tested. Some interspecific crosses showed evidence of resistance; PAR 1673-1 had less than 2% seed damage in 2002 and 2003 and less than 3% in 2005. PRA PRA 1142 sustained less than 3% seed damage and STR 1622-1 had less than 2% seed damage in three years of trials. Breeding lines with potential resistance included 01-4068-2, which had the least amount of seed damage per head in 2002 (<1%) and in 2003 averaged only 2% damage. Line 01-4080-1, with less than 1% damage in 2002 and in 2003, was the least damaged entry in these evaluations. Hybrid '894' was included as a standard check; however, it consistently had among the lowest average seed damage from *H. electellum* feeding. Our investigation showed the potential for developing resistant genotypes for the sunflower moth to reduce seed feeding injury and to prevent yield losses for sunflower producers. The development of germplasm with host plant resistance would provide another tool in an integrated pest management approach for *H. electellum*. Additional effort is in progress to use the identified lines to introgress resistance genes into cultivated sunflower through conventional breeding facilitated by marker-assisted selection.

**KEY WORDS** Cultivated sunflower, *Helianthus annuus*, pest management, host plant resistance, sunflower moth, *Homoeosoma electellum*

The sunflower moth, *Homoeosoma electellum* (Hulst) (Lepidoptera: Pyralidae), has been the most widespread and damaging insect pest of sunflower, *Helianthus annuus* L. (Asteraceae), in North America (Schulz 1978, Rogers 1988, Charlet et al. 1997). The moth occurs from Mexico to both coasts of the United States and to the Canadian Prairie Provinces (Chippendale & Cassatt 1986). Larval feeding has been reported on more than 40 different composite plant species including four species of native sunflowers, *Helianthus debilis* Nuttall, *H. maximiliani* Schrader, *H. petiolaris* Nuttall, and *H. tuberosus* L. (Teetes & Randolph 1969,

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Chippendale & Cassatt 1986, DePew 1986, Goodson & Neunzig 1993). The northern limit of the sunflower moth is approximately 40°N latitude, beyond which it does not overwinter (Arthur 1978). However, moths are transported north on southerly winds to the northern Plains of the United States and the Canadian Provinces of Saskatchewan and Manitoba (Arthur & Bauer 1981, Rogers & Underhill 1983).

Eggs are deposited on the surface of open sunflower heads. First instars feed primarily on pollen. Second instars feed on pollen and disk flowers. Feeding by third instars may sever the style preventing the ovary from being fertilized, resulting in empty seeds. Third instars also feed on the kernel of mature seeds. Larval feeding to maturity results in an average of about 96 damaged disk flowers and about 23 damaged ovaries per larva (Rogers 1978). As they feed, larvae spin a web over the face of the sunflower head, which accumulates destroyed disk flowers and frass, giving the head a trashy appearance. Larval feeding in the head also may provide a site for entrance of the fungal pathogen of *Rhizopus* head rot, which can further reduce yield and affect oil quality. Larvae exit the sunflower head when mature and drop into the soil to overwinter in silken cocoons covered with soil particles (Rogers 1978, 1992, Rogers & Westbrook 1985, Charlet et al. 1997).

Despite research on cultural and biological control and plant resistance, chemical control is frequently relied upon to manage sunflower moth infestations in commercial sunflower (Archer et al. 1983, Bynum et al. 1985, DePew 1988, Charlet et al. 1997). Although a large assemblage of tachinid and hymenopteran parasitoids have been reported to attack the sunflower moth in both agricultural and native sunflower habitats (Teetes & Randolph 1969, Beregovoy 1985, Charlet 1999, Chen & Welter 2002), control has often not been sufficient to reduce crop losses. Research by Chen & Welter (2007) revealed that larval densities were much lower and parasitism was higher on wild sunflowers than on cultivated sunflowers, because domesticated sunflower heads provided a structural refuge for the larvae from parasitoids. In Kansas, Aslam & Wilde (1991) showed that early June plantings usually had higher infestations than later plantings. Early studies showed that phytomelanin, a hard acellular layer that develops between the hypodermis and sclerenchyma in the pericarp of some sunflower lines, imparts mechanical resistance to the sunflower moth (Rogers & Kreitner 1983). Spring et al. (1987) found that sunflower species have simple, noncapitate glandular, and capitate glandular trichomes, with the capitate trichomes producing at least six different sesquiterpene lactones. Research showed that sesquiterpene lactones were feeding deterrents and toxins to the sunflower moth (Gershenzon et al. 1985, Rogers et al. 1987). Other sunflower compounds may also affect sunflower moth development; Elliger et al. (1976) and Rogers et al. (1987) found that sunflower diterpenes in artificial diet resulted in reduced larval performance. Although Rogers et al. (1984) released three germplasm lines for resistance to sunflower moth, there has been limited recent effort to evaluate lines for reduced seed injury by *H. electellum* in cultivated sunflower. Studies were initiated in 2002 to evaluate sunflower germplasm for potential resistance to the sunflower moth. Diverse sunflower germplasm was exposed to naturally occurring moth infestations to evaluate differences in seed damage caused by this insect pest.

## Materials and Methods

During the 2002 to 2007 growing seasons, 42 sunflower accessions (Plant Introductions [PI]), 25 breeding lines in early generation selection for resistance to the banded sunflower moth, *Cochylis hospes* Walsingham (Lepidoptera: Tortricidae), and 40 interspecific crosses derived from ten annual and five perennial *Helianthus* species were evaluated for resistance to infestation by naturally occurring populations of the sunflower moth. Each year, USDA sunflower Hybrid '894' was included in the trials because of its historical use as a standard check. Sunflower accessions were obtained from the USDA-ARS North Central Regional Plant Introduction Station, Ames, IA. The USDA, ARS Germplasm Resources Information Network (GRIN) online database was used to select accessions. Descriptors in the database were used to select lines with similar days to flowering and plant height. Interspecific crosses were provided by one of the authors (G.J.S.). Trials were conducted at the Northwest Research Extension Center, Kansas State University, Colby, Kansas. Twenty-one to 59 entries were evaluated annually in single row plots that were 8-m long. Rows were 76 cm apart, and plants were spaced 30.5 cm apart within rows, so that there were approximately 54,000 plants/ha. Entries with relatively low levels of seed damage per head along with some susceptible lines were selected for retesting in subsequent years. Plots were planted between 7 and 10 May each year in a randomized complete block design with four replicates, except for 2005 to 2007 when only three replicates were examined. Plots received a preplant application of fertilizer and herbicide, but no other chemical treatments were used.

Five heads per row (total of 15–20 heads per treatment) were removed after plants had senesced. Sunflower heads were harvested from late August to early September and sent to Fargo, ND. Heads were dried, threshed, and the seed cleaned prior to evaluation. Subsamples of 100 seeds per head were randomly selected and evaluated for number of seeds damaged by moth larval feeding. The degree of infestation was the percentage of seeds with *H. electellum* feeding injury per head. Visual examination of the pericarp was found to be an effective and reliable method to distinguish damage by the sunflower moth from other important seed feeding pests including the banded sunflower moth and red sunflower seed weevil, *Smicronyx fulvus* LeConte (Coleoptera: Curculionidae) (Peng & Brewer 1995).

The PROC GLM analysis of variance procedure (SAS 2008) was used to compare percentage of seed damaged per head among the different treatments for each study year. Percentages were transformed to the square root of the arcsine prior to analysis. Means were separated using the Fisher's protected least significant difference (LSD) test (Carmer & Walker 1985) at $P < 0.05$.

## Results

The determination of feeding damage in 2002 showed high levels of sunflower moth infestation within the trial based on the percentage of damaged seeds in individual heads sampled. The percentage of *H. electellum* seed damage per head ranged from 0 to 73% in the individual heads evaluated. The mean larval seed damage varied from 1 to 22% among the germplasm tested and the data did show

significant differences among a number of those tested (Table 1). Twenty four of the 59 lines included in the study showed less than 2% seed damage. Among the material in the trial, those less than 1% damage included the four lines 01-4059-1, 01-4043-1, 01-4080-1, 01-4068-2; the interspecific crosses RF ANN 1742, PRA-HIR 437, PRA RUN 417-1, ANO 1509-1; accession PI 243078; and Hybrid 894. The line 01-4094-1 was the most susceptible entry (22%) in 2002.

High levels of sunflower moth infestation occurred within the 2003 trial, based on the percentage of seed damage to individual heads. The percentage of *H. electellum* seed damage ranged from an average of 0.2 to 47% (Table 1). The line 01-4094-1 was again the most susceptible of all material in the trial. Seven of the 54 lines in the study, including Hybrid 894, had 2.2% or less seed damage per head. Although statistical differences were not always clearly defined, these lines were significantly different from over 25% of the germplasm tested. Resistant lines included the interspecific crosses HIR 1734-1, PAR 1673-1, and STR1622-2; the accessions PI 170414 and PI 372259; and the line 01-4080-1 (less than 1% damage in the 2002 trial).

There was a reduced level of infestation of *H. electellum* in the 2004 trial, which was reflected in a low amount of feeding damage among all the germplasm tested. The percentage of seed damage per head from *H. electellum* larval feeding ranged from 0 to 2% in the 36 lines tested. Because of the low level of damage from sunflower moth feeding, it was difficult to make meaningful comparisons among the germplasm tested. Thus, the trial was repeated in 2005 with the same lines.

In 2005, only two germplasm lines had over 36% damage, while the remaining 34 lines showed an average of about 10% or less *H. electellum* seed damage per head (Table 1). Although some inconsistencies in the results were evident compared to those in previous years, a number of lines that had shown low damage levels in 2002 and 2003 also had a low percentage of seed damage per head in 2005. The susceptible line 01-4094-1 was again the most heavily damaged (42% of seeds damaged) by sunflower moth feeding. HIR 1734-1 had over 36% damaged seed per head in 2005, but averaged only 2% damage per head in 2003. Four lines with low damage (≤2%) in 2003 also sustained an average of 2% or less damage per head in 2005 and were statistically lower than 25% of the germplasm in the trial. These lines included Hybrid 894, the accessions PI 170414 and PI 372259, and the interspecific cross STR 1622-2. Nine accessions that were new in the 2005 trial also had less than 2% seed damage per head from sunflower moth feeding, and of these, three averaged 1% or less damage per head: PI 193775, PI 494861, and PI 650497.

Insect pressure from the sunflower moth was very heavy in 2006 as shown by the amount of seed damage in the trial; the mean ranged from 1 to 80% seed damage among the selected germplasm lines evaluated (Table 1). The amount of damage sustained by the germplasm tested was surprising because, other than the susceptible checks, lines included in the 2006 trial had shown 4% or less damage in 2005. The interspecific cross HIR 1734-3 sustained an average of 56% seed damage per head in 2006, but had only 1% and 4% damage per head in 2005 and 2003, respectively. However, Hybrid 894 again had the lowest amount of seed damage per head in 2006, which is consistent with results from 2002, 2003, and 2005. During all four years, this line sustained no more than 2.2% damage per head, which was significantly lower than eight of the 22 lines evaluated. Others

in the 2006 trial with low seed damage levels included PI 170414 (10.6%), PI 170385 (10.7%), and PI 650375 (10.9%), which averaged 0%, 2.5%, and 1.1% damage, respectively, in 2005.

There was a high infestation pressure of *H. electellum* in 2007 based upon the amount of seed damage in the trial; the damage ranged from 8 to 82% seed damage per head among the 21 selected accessions, interspecific crosses, and the hybrid evaluated (Table 1). This year had the highest mean in the six years of evaluations with an average of 43% seed damage per head. Even though Hybrid 894 sustained 14% damage in 2007, this was still among the lowest damage levels and was statistically lower than eight of the lines tested. PI 170414 consistently had one of the lowest levels of damage from *H. electellum* with an average of 9.3% damage in 2007, 10.6% damage in 2006 and 0% damage in 2005. The accession with the lowest damage in 2007 was PI 177399 with only 8.8% feeding damage per head. However, this was the only year this accession was tested.

## Discussion

Germplasm with resistance to attack and damage from larval feeding by the sunflower moth was evident from this six-year study, although in some cases differences were not clearly defined statistically. It is likely that large variation among plots, as reflected by large standard errors, reduced the statistical significance among lines. PI 175728 and PI 307946 both showed less than 3% feeding damage per head in three years they were tested. However, these lines were not tested during 2006 and 2007 when high populations of *H. electellum* were present and seed damage averaged 32% and 43%, respectively. PI 170414 exhibited less than 1% feeding damage per head in two years of trials, but suffered over 10% damage in 2006 and 9% in 2007. Two lines (PI 170401 and PI 372259) that appeared promising in both 2003 and 2005 with less than 3% seed damage per head, were heavily damaged in the final two trial years. This reaction could possibly be because resistance mechanisms were overwhelmed in 2006 and 2007. Additional research would be needed to determine whether mechanisms such as antibiosis or antixenosis were responsible for the resistance and broke down due to high *H. electellum* population pressure. PI 177399 may have potential because it significantly had the least damage in 2007 among all germplasm tested; however, this was the only year of testing for this line.

A number of interspecific crosses showed evidence of resistance in three years of trials. PAR 1673-1 (*H. paradoxus* Heiser) had less than 2% seed damage per head in both 2002 and 2003 and less than 3% in 2005. PRA PRA 1142 (*H. praecox* Engleman and Gray) also sustained less than 3% seed damage per head in three years of testing (2002, 2003, and 2005), and STR 1622-1 (*H. strumosus* L.) showed less than 2% seed damage per head for the same three years. However, another selection from this interspecific cross, STR 1622-2 (*H. strumosus*) had inconsistent results; it had only 1% seed damage per head in 2003 and 2005, but over 20% in the subsequent two years of trials due to high populations of *H. electellum*. Two other interspecific crosses, DEB CUC 1810 (*H. debilis* Nuttall ssp. *cucumerifolius* (Torrey & Gray) Heiser) and HIR 1734-3 (*H. hirsutus* Rafinesque) had similar conflicting results with low damage in two of four years of testing.

**Table 1. Percentage of seeds damaged by *Homoeosoma electellum* in selected sunflower accessions evaluated at Colby, KS, 2002 to 2007.**

| | Percent damaged seed per head (mean ± SE) | | | | |
|---|---|---|---|---|---|
| Accession | 2002 | 2003 | 2005[a] | 2006 | 2007 |
| PI 162453 | — | — | 3.8 ± 0.2cdefghi | 53.3 ± 15.0abcde | — |
| PI 162675 | — | 16.5 ± 6.3bcdef | — | — | — |
| PI 167387 | 1.4 ± 0.4hijkl | 19.4 ± 2.8b | 2.7 ± 1.3defghijk | — | — |
| PI 170385 | — | — | 2.5 ± 1.3defghijk | 10.7 ± 7.8fg | 65.9 ± 18.9abcd |
| PI 170387 | 3.2 ± 0.8defghijkl | — | — | — | — |
| PI 170391 | — | 5.4 ± 4.2fghijkl | — | — | — |
| PI 170399 | 1.8 ± 0.8ghijkl | 6.0 ± 2.9cdefghijkl | — | — | — |
| PI 170401 | — | 2.7 ± 0.8hijkl | 2.0 ± 1.0efghijk | 13.6 ± 7.1fg | 62.4 ± 15.1abcd |
| PI 170405 | — | — | 1.4 ± 0.4fghijk | 21.5 ± 12.8cdefg | 23.7 ± 2.0efg |
| PI 170412 | 4.0 ± 2.5defghijkl | — | — | — | — |
| PI 170414 | — | 0.9 ± 0.7kl | 0.0k | 10.6 ± 4.8fg | 9.3 ± 6.5fg |
| PI 170415 | — | 3.9 ± 1.6ghijkl | 2.9 ± 0.9defghijk | 18.5 ± 14.9cdefg | 13.3 ± 5.5efg |
| PI 170417 | 11.0 ± 3.7b | — | — | — | — |
| PI 170418 | — | — | 5.3 ± 0.9bcdefg | — | — |
| PI 170419 | — | 17.4 ± 7.5bcde | — | — | — |
| PI 175728 | 1.9 ± 1.0fghijkl | 2.7 ± 1.2hijkl | 1.3 ± 0.5ghijk | — | — |
| PI 177396 | — | — | — | — | 64.3 ± 9.9abcd |
| PI 177399 | — | — | — | — | 8.8 ± 6.5g |
| PI 193775 | — | — | 0.5 ± 0.2ijk | 28.2 ± 12.5bcdefg | 41.8 ± 9.3cdef |
| PI 232905 | 3.0 ± 2.4fghijkl | — | — | — | — |
| PI 243078 | 0.8 ± 0.4kl | 7.0 ± 3.0defghijkl | 5.7 ± 2.0bcdef | — | — |
| PI 250855 | 9.6 ± 2.9bc | — | — | — | — |
| PI 251902 | 1.9 ± 0.6fghijkl | 5.8 ± 2.5cdefghijkl | — | — | — |
| PI 253773 | 2.1 ± 0.6fghijkl | — | — | — | — |
| PI 253776 | — | — | 9.5 ± 3.5bc | — | — |

**Table 1. Continued.**

| Accession | Percent damaged seed per head (mean ± SE) | | | | |
|---|---|---|---|---|---|
| | 2002 | 2003 | 2005[a] | 2006 | 2007 |
| PI 267665 | — | — | 10.5 ± 4.0b | — | — |
| PI 287233 | — | — | — | — | 24.1 ± 5.1efg |
| PI 291403 | — | — | 1.8 ± 0.8efghijk | 26.4 ± 4.5bcdefg | 72.5 ± 2.7abcd |
| PI 291407 | — | — | — | — | 76.3 ± 9.2ab |
| PI 307946 | 1.7 ± 0.8ghijkl | 2.3 ± 0.5ghijkl | 0.5 ± 0.3hijk | — | — |
| PI 343785 | 2.0 ± 0.5fghijkl | 9.5 ± 4.1cdefghijkl | — | — | — |
| PI 372259 | — | 1.5 ± 0.9jkl | 0.8 ± 0.5hijk | 38.1 ± 21.8bcdef | 46.4 ± 14.8abcde |
| PI 386230 | — | — | 5.4 ± 3.3cdefghi | — | — |
| PI 431516 | — | — | 2.0 ± 2.0cdefghij | 79.8 ± 13.6a | — |
| PI 431542 | — | — | 6.7 ± 3.2bcde | — | — |
| PI 494859 | — | — | 1.3 ± 1.0hijk | 60.1 ± 11.5ab | — |
| PI 494861 | — | — | 0.5 ± 0.2ijk | 15.8 ± 6.7efg | 43.5 ± 17.3bcde |
| PI 497939 | — | — | 6.5 ± 4.5bcd | — | — |
| PI 505651 | — | — | 1.6 ± 0.2efghijk | 33.4 ± 17.3bcdefg | 39.8 ± 4.8def |
| PI 650375 | — | — | 1.1 ± 0.5ghijk | 10.9 ± 6.1fg | 19.2 ± 7.2efg |
| PI 650497 | — | — | 1.0 ± 0.5ghijk | 43.3 ± 21.0bcdef | 21.1 ± 8.6efg |
| PI 650558 | — | — | 5.8 ± 5.8cdefgh | 56.2 ± 2.4abcd | — |
| ANO 1509-1 | 0.7 ± 0.4jkl | 3.0 ± 2.6hijkl | — | — | — |
| ANO 1509-2 | — | 3.0 ± 2.8ijkl | — | — | — |
| ARG 420-1 | 3.8 ± 2.0defghijkl | — | — | — | — |
| ARG 1575-2 | 2.2 ± 1.3fghijkl | — | — | — | — |
| ARG 1575-4 | — | 6.6 ± 2.7bcdefghijkl | — | — | — |
| BOL 774 | 4.8 ± 1.2bcdef | — | — | — | — |
| DEB CUC 1810 | — | 2.9 ± 1.3ghijkl | 0.7 ± 0.5hijk | 20.3 ± 3.6cdefg | 81.5 ± 6.4a |
| DEB SIL 367-2 | 3.3 ± 2.6ghijkl | — | — | — | — |
| DES 1474-2 | 3.0 ± 1.4defghijkl | — | — | — | — |
| DES 1474-3 | — | 16.8 ± 8.8bc | — | — | — |

**Table 1. Continued.**

| Accession | Percent damaged seed per head (mean ± SE) | | | | |
|---|---|---|---|---|---|
| | 2002 | 2003 | 2005[a] | 2006 | 2007 |
| GIG 1616-2 | 3.3 ± 1.6defghijk | — | — | — | — |
| HIR 1734-1 | — | 2.0 ± 0.8hijkl | 36.5 ± 11.5a | 31.1 ± 13.5bcdefg | 72.3 ± 26.0abc |
| HIR 1734-3 | — | 4.3 ± 1.7cdefghijkl | 1.0 ± 1.0fghijk | 55.7 ± 27.6abc | 78.1 ± 21.9a |
| NEG 1255 | 3.1 ± 2.0defghijkl | — | — | — | — |
| PAR 1084-1 | 5.0 ± 4.0cdefghijkl | — | — | — | — |
| PAR 1671-1 | 1.9 ± 0.8ghijkl | 16.8 ± 5.1b | — | — | — |
| PAR 1673-1 | 1.4 ± 0.6hijkl | 1.7 ± 0.2jkl | 2.6 ± 1.7efghijk | — | — |
| PAR 1673-2 | — | 10.8 ± 4.1bcdefghij | — | — | — |
| PET PET 1741-2 | 5.9 ± 2.6cdefgh | — | — | — | — |
| PRA HIR 437 | 0.8 ± 0.3jkl | 8.4 ± 1.6bcdefghij | — | — | — |
| PRA PRA 1142 | 1.9 ± 0.7ghijkl | 2.7 ± 1.2hijkl | 1.7 ± 1.2ghijk | — | — |
| PRA RUN 417-1 | 0.6 ± 0.3kl | 7.2 ± 3.1bcdefghijkl | — | — | — |
| PRA RUN 1329 | — | 7.6 ± 1.9bcdefghijk | — | — | — |
| RES 834-1 | — | 11.0 ± 3.8bcdefghi | — | — | — |
| RES 834-3 | — | 7.4 ± 2.6bcdefghijk | — | — | — |
| RF ANN 19 | — | 10.9 ± 5.0bcdefgh | — | — | — |
| RF ANN 48 | — | 8.2 ± 6.2bcdefghijkl | — | — | — |
| RF ANN 783 | — | 5.9 ± 3.6cdefghijkl | — | — | — |
| RF ANN 892 | — | 6.3 ± 0.7bcdefghijk | — | — | — |
| RF ANN 1064 | — | 5.5 ± 3.1defghijkl | — | — | — |
| RF ANN 1742 | 0.9 ± 0.5jkl | 15.8 ± 5.0bcde | — | — | — |
| RF ARG 420 | — | 16.6 ± 9.6bcd | — | — | — |
| RF ARG 1575 | — | 13.7 ± 3.4bcd | — | — | — |
| RF PRA 417 | — | 16.4 ± 5.3bcdefg | — | — | — |
| RF TUB 346 | — | 13.2 ± 2.5bcd | — | — | — |
| STR 1622-1 | 1.3 ± 0.5ghjkl | 2.3 ± 1.0hijkl | 1.5 ± 0.9efghijk | — | — |

**Table 1. Continued.**

| Accession | Percent damaged seed per head (mean ± SE) | | | | |
|---|---|---|---|---|---|
| | 2002 | 2003 | 2005[a] | 2006 | 2007 |
| STR 1622-2 | — | 1.3 ± 0.3jkl | 1.0 ± 0.5ghijk | 20.4 ± 19.9defg | 23.9 ± 5.8efg |
| TUB 346 | 2.6 ± 0.4defghijkl | — | — | — | — |
| TUB 365 | 1.8 ± 0.3defghijkl | 10.6 ± 6.7bcdefghij | — | — | — |
| TUB 1709-1 | 4.5 ± 1.6cdefghi | — | — | — | — |
| 01-4023-1 | 1.4 ± 0.8hijk | 8.7 ± 3.6bcdefghijk | — | | |
| 01-4027-2 | 4.1 ± 1.1cdefgh | — | — | — | — |
| 01-4039-2 | 21.5 ± 3.0a | 42.1 ± 6.4a | 8.4 ± 1.3bcd | — | — |
| 01-4043-1 | 0.9 ± 0.6kl | — | — | — | — |
| 01-4047-1 | 2.0 ± 1.4ghijkl | — | — | — | — |
| 01-4048-2 | 2.2 ± 0.9defghijkl | 8.9 ± 3.3bcdefghijk | — | — | — |
| 01-4050-1 | 3.1 ± 1.6defghijkl | — | — | — | — |
| 01-4051-1 | 5.0 ± 2.2cdefghi | — | — | — | — |
| 01-4056-2 | 2.7 ± 1.0efghijkl | — | — | — | — |
| 01-4058-3 | 2.1 ± 1.4ghijkl | — | — | — | — |
| 01-4059-1 | 0.9 ± 0.4ijkl | 4.0 ± 1.3efghijkl | — | — | — |
| 01-4060-3 | 3.5 ± 0.3cdefghij | — | — | — | — |
| 01-4061-2 | 3.7 ± 1.0defghijkl | — | — | — | — |
| 01-4062-1 | 1.8 ± 0.6hijkl | 4.6 ± 2.9cdefghijkl | — | — | — |
| 01-4063-1 | 1.8 ± 0.5fghijkl | 3.2 ± 0.9defghijkl | — | — | — |
| 01-4066-2 | 3.0 ± 0.6defghijkl | — | — | — | — |
| 01-4068-2 | 0.7 ± 0.4l | 2.4 ± 1.3jkl | — | — | — |
| 01-4072-2 | 7.9 ± 3.6bcde | — | — | — | — |
| 01-4078-2 | 1.3 ± 1.2ijkl | 7.9 ± 2.9bcdefghijkl | — | — | — |
| 01-4080-1 | 0.8 ± 0.3hijkl | 0.2 ± 0.2l | — | — | — |
| 01-4085-2 | 2.0 ± 0.6fghijkl | — | — | — | — |
| 01-4089-1 | 10.2 ± 5.2bcd | — | — | — | — |
| 01-4090-1 | 5.9 ± 2.1bcdefg | — | — | — | — |

**Table 1. Continued.**

| Accession | Percent damaged seed per head (mean ± SE) | | | | |
|---|---|---|---|---|---|
| | 2002 | 2003 | 2005[a] | 2006 | 2007 |
| 01-4094-1 | 22.3 ± 10.1a | 46.5 ± 19.1a | 42.2 ± 6.5a | 47.3 ± 10.3abcdef | — |
| 01-4097-1 | 2.2 ± 1.0fghijkl | — | — | — | — |
| Hybrid 894 | 0.7 ± 0.2jkl | 2.2 ± 1.0jkl | 0.3 ± 0.1k | 1.2 ± 0.4g | 14.0 ± 5.0efg |
| Mean | 3.6 | 8.8 | 4.5 | 31.7 | 43.0 |

Means followed by the same letter within each year are not significantly different ($P < 0.05$; LSD); percentages transformed to square root of the arcsine before analysis and untransformed means are presented; 2–67 heads examined per accession each year.
[a]2004 data were not included because of reduced infestation levels.

Comparison of breeding lines evaluated in 2002 and 2003 revealed some potential resistant germplasm. The line 01-4068-2 had the least amount of seed damage per head in the 2002 trial with less than 1%, and in the next year averaged only 2% damage, while 01-4080-1 sustained less than 1% the first year and in 2003 was the lowest in the trial at 0.2% seed damage per head. The line 01-4094-1 was used as a susceptible check based on results from 2002 when it had the highest level of damage in the trial (22% seed damage per head). In the four years (2002, 2003, 2005, and 2006) that 01-4094-1 was included in the trials it sustained the greatest amount of *H. electellum* damage in three of those years.

Hybrid 894 was included in these trials as a standard check. It is a public-domain hybrid that has been produced by a number of commercial sources. In the past, it was used as a susceptible check in research studies for another sunflower pest, the banded sunflower moth (Brewer and Charlet 1989, Jyoti and Brewer 1999). However, in the current investigation, this hybrid consistently had among the lowest average seed damage from *H. electellum* feeding. In the first five years of evaluation, it had less than 2.2% seed damage each year, and it had among the lowest levels of seed damage in the final trial (2007) when overall damage levels were higher than in other years. In screening trials for resistance to stem-infesting pests, Hybrid 894 was not very resistant to attack by the sunflower stem weevil, *Cylindrocopturus adspersus* (LeConte) (Coleoptera: Curculionidae), a longhorned beetle, *Dectes texanus* LeConte (Coleoptera: Cerambycidae), or a root boring moth, *Pelochrista womonana* (Kearfott) (Lepidoptera: Tortricidae) (Charlet et al. 2009). However, in another study in which germplasm was evaluated for resistance to the banded sunflower moth, Hybrid 894 was also the most resistant line (Charlet et al. 2010).

Our investigation showed potential for developing sunflower moth-resistant genotypes that would reduce seed feeding injury, prevent yield loss, and increase grower profit. Host plant resistance would provide another tool in an integrated pest management approach for *H. electellum*. Although chemical control has been beneficial (Archer et al. 1983, Bynum et al. 1985, DePew 1988), it can be expensive and relies on field monitoring to be effective. An added benefit of host plant resistance is that it can be effectively combined with delayed planting, which has also been shown to reduce densities of *H. electellum* and reduce crop losses (Aslam & Wilde 1991). In addition, reduced chemical treatments would be less detrimental to the natural enemies of the sunflower moth (Teetes & Randolph 1969, Beregovoy 1985, Charlet 1999). The nature of the resistance mechanisms resulting in the reduced seed damage in the germplasm is not known, but will be the subject of future research. The resistance may be due to phytomelanin (Rogers et al. 1992), sesquiterpene (Gershenson et al. 1985, Rogers et al. 1987, Spring et al. 1987), or diterpene (Elliger et al. 1976, Rogers et al. 1987) feeding deterrents. Additional effort is in progress to use the identified lines to introgress resistance genes into cultivated sunflower through conventional breeding facilitated by marker-assisted selection.

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# Resistance in Cultivated Sunflower to the Sunflower Moth (Lepidoptera: Pyralidae)[1]

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J. Agric. Urban Entomol. 25(4): 245–257 (October 2008)

**ABSTRACT** A five-year field study evaluated 42 sunflower (*Helianthus annuus* L.) accessions, 25 breeding lines, and 40 interspecific crosses for resistance to infestation and damage from larval feeding by naturally occurring populations of the sunflower moth, *Homoeosoma electellum* (Hulst) (Lepidoptera: Pyralidae). Accessions PI 175728 and PI 307946 had less than 3% feeding damage per head in all three years they were tested. Some interspecific crosses showed evidence of resistance; PAR 1673-1 had less than 2% seed damage in 2002 and 2003 and less than 3% in 2005. PRA PRA 1142 sustained less than 3% seed damage and STR 1622-1 had less than 2% seed damage in three years of trials. Breeding lines with potential resistance included 01-4068-2, which had the least amount of seed damage per head in 2002 (<1%) and in 2003 averaged only 2% damage. Line 01-4080-1, with less than 1% damage in 2002 and in 2003, was the least damaged entry in these evaluations. Hybrid '894' was included as a standard check; however, it consistently had among the lowest average seed damage from *H. electellum* feeding. Our investigation showed the potential for developing resistant genotypes for the sunflower moth to reduce seed feeding injury and to prevent yield losses for sunflower producers. The development of germplasm with host plant resistance would provide another tool in an integrated pest management approach for *H. electellum*. Additional effort is in progress to use the identified lines to introgress resistance genes into cultivated sunflower through conventional breeding facilitated by marker-assisted selection.

**KEY WORDS** Cultivated sunflower, *Helianthus annuus*, pest management, host plant resistance, sunflower moth, *Homoeosoma electellum*

The sunflower moth, *Homoeosoma electellum* (Hulst) (Lepidoptera: Pyralidae), has been the most widespread and damaging insect pest of sunflower, *Helianthus annuus* L. (Asteraceae), in North America (Schulz 1978, Rogers 1988, Charlet et al. 1997). The moth occurs from Mexico to both coasts of the United States and to the Canadian Prairie Provinces (Chippendale & Cassatt 1986). Larval feeding has been reported on more than 40 different composite plant species including four species of native sunflowers, *Helianthus debilis* Nuttall, *H. maximiliani* Schrader, *H. petiolaris* Nuttall, and *H. tuberosus* L. (Teetes & Randolph 1969,

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SCIENTIFIC NOTE

# Genetic diversity of field populations of the cat flea, *Ctenocephalides felis*, and the human flea, *Pulex irritans*, in the South Central United States[1]

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**ABSTRACT** The cat flea, *Ctenocephalides felis*, and the human flea, *Pulex irritans*, are species of significant human and veterinary health concerns. Genetic analysis of these species may provide insight into the dispersal of these insects and the spread of insecticide resistance. For this study, a total of 58 fleas were collected from Texas and Arkansas from both dwellings and pet animals. A total of 52 fleas collected were identified as *C. felis*, and 6 were identified as *P. irritans*. Samples were subjected to PCR and DNA sequencing using two markers, a portion of the mitochondrial DNA 16S rRNA gene and the nuclear rRNA first internal transcribed spacer region. For both species, no genetic variation was observed in either the nuclear or mitochondrial markers. Based on this observed lack of genetic variation, *C. felis* and *P. irritans* possibly underwent a genetic bottleneck in the United States.

**KEY WORDS** cat flea, human flea, DNA sequence, genetic variation

The most common domestic flea in the United States is the cat flea, *Ctenocephalides felis* (Bouché). It can reproduce on both dogs and cats and is the most important ectoparasite of these species worldwide (Rust & Dryden 1997). The human flea, *Pulex irritans* L., is associated with humans as well as with wild mammals such as foxes, skunks and coyotes. This species is thought to have originated in South America, where its original host may have been the guinea pig or peccary that were living in close proximity to human dwellings (Buckland & Sadler 1989). It is one of six species in the genus *Pulex*; the other five are confined to the Nearctic and Neotropical regions (Whiting et al. 2008). Often, fleas can become established in dwellings where pet animals are infested, and then they will bite humans. In order to effectively control an infestation, fleas must be removed from the pet, the home, and the surrounding area. Over one billion dollars are spent annually by pet owners in the USA for flea control

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**Table 1. Sample collection data and haplotype for *Ctenocephalides felis* and *Pulex irritans* found in this study.**

| Species | State | County | Haplotype (n) |
|---|---|---|---|
| *Ctenocephalides felis* | AR | Benton | F1(1) |
| | | Jasper | F1(1) |
| | | Johnson | F1(1) |
| | | Phillips | F1(2) |
| | | Saline | F1(1) |
| | | Washington | F1(5) |
| | TX | Bexar | F1(1) |
| | | Brazos | F1(29) |
| | | Cherokee | F1(1) |
| | | Gregg | F1(2) |
| | | Harris | F1(2) |
| | | Kaufman | F1(1) |
| | | Montgomery | F1(1) |
| | | Nueces | F1(1) |
| | | Travis | F1(1) |
| | | Waller | F1(1) |
| | | Wharton | F1(1) |
| *Pulex irritans* | TX | Brazos | F2(4) |
| | | Cherokee | F2(1) |
| | | Wichita | F2(1) |

(MacAllister 1993). The cat flea has developed insecticide resistance to at least five different classes of insecticides (WHO 1992).

Knowledge of genetic variation within medically important insect species is an essential element required for understanding vector transmission, disease epidemiology, and disease control (Tabachnick & Black 1995). Because nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) are inherited by independent evolutionary means, their combined application to elucidate gene flow of fleas can reveal both their sexually and matrilineally derived ancestry. A previous study by Vobis et al. (2004) was conducted on *C. felis* populations from the United States and Europe using DNA sequencing analysis of the nDNA ribosomal first and second internal transcribed spacer regions (ITS1 and ITS2), and the mtDNA rRNA 16S gene. The study by Vobis et al. (2004), which focused on lab colonies from the United States, did not find any genetic variation for either marker. Therefore, the objective of this study was to determine the extent of genetic variation within and among field populations of *C. felis* and *P. irritans* from the south-central United States using both mitochondrial and nuclear DNA markers.

## Materials and Methods

Flea specimens were collected from Arkansas and Texas in 2007 and 2008 (Table 1). Samples were morphologically identified to species using keys by Furman & Catts (1986). Voucher specimens preserved in 100% ethanol are

maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville, AR.

DNA was extracted from individual fleas using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μl of Tris:EDTA and stored at −20°C. Polymerase chain reaction was conducted using the primers LR-J-13007 (5′-TTACGCTGTTATCCCTAA-3′) (Kambhampati & Smith 1995) and LR-N-13398 (5′-CGCCTGTTTATCAAAAA CAT-3′) (Simon et al. 1994). These PCR primers amplify an approximately 428 bp region of the mtDNA 16S rRNA gene. The PCR reactions were conducted with 1 μl of the extracted DNA (Szalanski et al. 2000), having a profile consisting of 35 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 60 s for the 16S marker. PCR was also conducted using the primers rDNA1.58s and rDNA2 to amplify the small subunit RNA ITS marker. For the ITS1 maker, a 3′ portion of 18S rDNA, all of ITS1, and the 5′ portion of 5.8S was first amplified using the primers rDNA2 (5′-TTGATTACGTCCCTGCCCTTT-3′) (Vrain et al. 1992) and rDNA 1.58S (5′-GCCACCTAGTGAGCCGAGCA-3′) (Cherry et al. 1997) with a thermalcycler profile consisting of 40 cycles of 94°C for 45 s, 53°C for 1 min, and 72°C for 1 min as described by Szalanski and Owens (2003). Amplified DNA from individual fleas was purified and concentrated with minicolumns (Wizard PCRpreps, Promega, Madison, WI) according to the manufacturer's instructions. Samples were sent to the University of Arkansas Medical Center DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions. GenBank accession numbers were GQ387496 to GQ387498 for the new sequences found in this study. Consensus sequences for each sample were obtained using Bioedit 5.09 (Hall 1999). Sequences were compared with ones available on GenBank using BLAST search (www.ncbi.nlm.nih.gov/blast/, which is an algorithm for comparing the similarity of DNA sequences).

## Results and Discussion

Of the 58 fleas that were subjected to DNA sequencing analysis, 52 were *C. felis* and the remainder were *P. irritans* (Table 1). The ITS1 marker was 948 bp in size for *P. irritans* and 830 bp in size for *C. felis*. No intraspecific variation was observed for either species for the ITS1 marker. Using a BLAST search, the *P. irritans* ITS1 sequence was 89% similar to *P. irritans* from Cameroon (GenBank accession number EU169198), and the *C. felis* ITS1 sequence was identical to *C. felis* from Cameroon (GenBank accession number EU170156).

The mtDNA 16S marker was 419 bp in size for both *C. felis* and *P. irritans*. As with the nuclear rRNA ITS1 marker, no intraspecific variation was observed for either species. No DNA sequences for this marker in *C. felis* or *P. irritans* exist on GenBank, so a BLAST search was not preformed.

Vobis et al. (2004) found little variation in cat flea DNA collected from Europe and from laboratory colonies from the United States using both the mtDNA 16S marker and the nuclear ITS1 and ITS2 markers. Vobis et al. (2004) also studied a population of *P. irritans* from Austria and found no intraspecific genetic variation. We also found a lack of genetic variation from our field collections of *C. felis* and *P. irritans* from Arkansas and Texas. Unfortunately, the DNA sequences from Vobis et al. (2004) were not deposited to GenBank, so comparisons of our data with theirs cannot be made. Molecular genetic variation

studies of other Siphonaptera are limited. The only other species that has been subjected to DNA sequencing analysis is *Tunga penetrans* (L.) from South America and Africa populations using the mtDNA cytochrome oxidase II and ITS2 markers (Luchetti et al. 2007). As with *C. felis* and *P. irritans*, low levels of mtDNA genetic variation were observed, while the ITS2 marker revealed a distinction between populations from Ecuador with those from Brazil or Africa. This difference in mitochondrial and nuclear genetic markers has also been observed for another blood-feeding insect, *Cimex lectularius* L. (Szalanski et al. 2008); however, with this bed bug, there was a large amount of mitochondrial genetic variation and a lack of ITS1 genetic variation, indirectly supporting suppositions about the chronology of reemerging populations of this pest.

Based on our results, field populations of *C. felis* and *P. irritans* in Arkansas and Texas have either undergone a genetic bottleneck due to insecticide pressure or they are a recent introduction. Some populations of *C. felis* that might constitute possible assemblages of resistance to insecticides such as imidacloprid, (Rust et al. 2002), carbaryl, chlorpyrifos, malathion, and PBO-synergized pyrethrin (Bass et al. 2004a,b, Bossard et al. 2002), have been reported. However, if insecticides are driving this genetic isolation, insect growth regulators (IGRs) may be the most likely candidates to affect this genetic shift due to their broad use by veterinarians and the easy access to consumers from the over-the-counter market segment of animal health products. This being stated, reports of insecticide resistance are few (Rust 2005). In the future, a more in-depth study involving microsatellite markers and a broader geographical sample may provide more insight into the genetic diversity of these two economically important flea species. For now, this is the first attempt in Texas or Arkansas to observe these public and animal nuisance pests from a genetic perspective. Future evaluation of IGR contribution to genetic isolation and resistance of these species is highly recommended.

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SCIENTIFIC NOTE

# Genetic diversity of field populations of the cat flea, *Ctenocephalides felis*, and the human flea, *Pulex irritans*, in the South Central United States[1]

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**ABSTRACT** The cat flea, *Ctenocephalides felis*, and the human flea, *Pulex irritans*, are species of significant human and veterinary health concerns. Genetic analysis of these species may provide insight into the dispersal of these insects and the spread of insecticide resistance. For this study, a total of 58 fleas were collected from Texas and Arkansas from both dwellings and pet animals. A total of 52 fleas collected were identified as *C. felis*, and 6 were identified as *P. irritans*. Samples were subjected to PCR and DNA sequencing using two markers, a portion of the mitochondrial DNA 16S rRNA gene and the nuclear rRNA first internal transcribed spacer region. For both species, no genetic variation was observed in either the nuclear or mitochondrial markers. Based on this observed lack of genetic variation, *C. felis* and *P. irritans* possibly underwent a genetic bottleneck in the United States.

**KEY WORDS** cat flea, human flea, DNA sequence, genetic variation

The most common domestic flea in the United States is the cat flea, *Ctenocephalides felis* (Bouché). It can reproduce on both dogs and cats and is the most important ectoparasite of these species worldwide (Rust & Dryden 1997). The human flea, *Pulex irritans* L., is associated with humans as well as with wild mammals such as foxes, skunks and coyotes. This species is thought to have originated in South America, where its original host may have been the guinea pig or peccary that were living in close proximity to human dwellings (Buckland & Sadler 1989). It is one of six species in the genus *Pulex*; the other five are confined to the Nearctic and Neotropical regions (Whiting et al. 2008). Often, fleas can become established in dwellings where pet animals are infested, and then they will bite humans. In order to effectively control an infestation, fleas must be removed from the pet, the home, and the surrounding area. Over one billion dollars are spent annually by pet owners in the USA for flea control

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# Assessment of *Chrysoperla plorabunda* Longevity, Fecundity, and Egg Viability When Adults Are Fed Transgenic *Bt* Corn Pollen[1]

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**ABSTRACT** The widespread planting of transgenic corn containing *Bacillus thuringiensis* (*Bt*) Cry endotoxin in its tissues for insect pest control raises the potential for influence on many non-target species including pollen-feeding species of Chrysopidae. This study was conducted to assess fitness parameters associated with longevity, fecundity, and egg viability of adult *Chrysoperla plorabunda* (Fitch) (Neuroptera: Chrysopidae) when fed *Bt* corn pollen. *Bt* products tested with their respective non-*Bt* near-isolines were Event 176 (Cry1Ab), MON810 (Cry1Ab), and TC1507 (Cry1F). Females fed pollen from Event 176 lived significantly longer than those fed pollen from its non-*Bt* near-isoline. Males fed pollen from TC1507 showed a trend for living longer than males fed its non-*Bt* near-isoline pollen, but there was no difference for females regarding this event. The mean number of eggs produced per female per day was significantly less for those fed MON810 pollen compared with females fed pollen from the non-*Bt* near-isoline. Total egg production was significantly less for females fed MON810 pollen vs. females fed pollen from its non-*Bt* near-isoline.

**KEY WORDS** lacewing, *Chrysoperla plorabunda*, *Bacillus thuringiensis*, transgenic corn, non-target insect, fitness

Bioengineered transgenic crops that were developed in the 1990s by incorporating *Bacillus thuringiensis* (*Bt*) Cry protein endotoxin directly into plant tissues to make them highly resistant to pest insects provide very effective pest control (Pedigo & Rice 2009). Incorporation of *Bt* Cry protein into corn through genetic engineering (Koziel et al. 1993) was readily commercialized and *Bt* corn acreage has substantially increased over the past decade. *Bt* corn was planted on 55 million acres in the USA in 2009, representing 63% of the total acreage planted to field corn that year (USDA-NASS 2009). This percentage includes varieties containing *Bt* only (17% of corn acreage) and stacked varieties (46% of corn acreage) containing both *Bt* and herbicide-resistance genes. Corn

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varieties containing genes for Cry1Ab are targeted primarily for European corn borer (Lepidoptera: Crambidae) in North America (Baute 2004).

Even before its release, concern was expressed regarding the impact of *Bt* corn tissue on non-target insects and other organisms. Wolfenbarger et al. (2008) provided a recent meta analysis of the effects of *Bt* corn on non-target arthropods. Among various non-target species, Andow & Hilbeck (2004, page 645) described *Chrysoperla carnea* [*sensu lato*] (Neuroptera: Chrysopidae) as a "high-priority secondary consumer for nontarget testing" that is "often included as a universal indicator species in ecotoxicological testing of pesticides." Until recently, the common green lacewing was known as *Chrysoperla carnea* (Stephens). The Old World form of the common green lacewing, now recognized as a distinct species, retained the name *Chrysoperla carnea*, while the New World form was re-named *Chrysoperla plorabunda* (Fitch) (Brooks 1994). *Chrysoperla plorabunda* is widely distributed in the USA and is the most common species in the corn belt region, whereas a related species *C. rufilabris* Burmeister, is more prevalent in the southeastern states. Differences in humidity between these areas may account for the geographic differences (Tauber & Tauber 1983).

Because of the beneficial nature of the predatory larvae of *C. plorabunda* in North America and its Eurasian counterpart, *C. carnea*, concern has been raised and extensive studies have been conducted to determine the effects of Cry protein on these species. To date, nearly all of the published studies have focused on the larval stages. Earlier works indicated possible effects due to direct and indirect exposure to *Bt* Cry protein (Salama et al. 1982, Pilcher et al. 1997, Hilbeck et al. 1998a,b, Lozzia et al. 1998, Dutton et al. 2002). However, more recent studies (Romeis et al. 2004, 2006, Rodrigo-Simón et al. 2006, Wolfenbarger et al. 2008) do not show significant detrimental effects of Cry proteins on *C. carnea* larvae when ingested either directly or indirectly after *Bt* containing tissue is consumed by prey. Obrist et al. (2006a) found that Cry1Ab toxin levels in first instar *C. carnea* corresponded with levels of Cry1Ab toxin present in two prey species, *Tetranychus urticae* Koch (Acari: Tetranychidae) and *Spodoptera littoralis* Biosduval (Lepidoptera: Noctuidae).

The impact of Cry protein on adult *Chrysoperla* spp. has received little attention until recently (Obrist et al. 2006b, Li et al. 2008). In contrast to larvae, the non-predatory adults are not likely to be exposed to Cry proteins through consumption of arthropod prey, because they generally develop and reproduce by feeding on pollen and glycolic substances (honeydew, nectar, and sugary exudates) (Sheldon & MacLeod 1971, 1974, Canard 2001). The common green lacewing in Illinois was reported to disperse in large numbers into corn fields during tasseling where it was observed feeding extensively on corn pollen (Sheldon & MacLeod 1971, 1974). The pollen provides proteins and other nutrients that facilitate egg development. However, if the pollen contains Cry proteins, it could affect adult development, survival, and fitness, which could lead to higher mortality than in non-*Bt* corn, or otherwise affect populations of this important insect predator over successive generations.

Obrist et al. (2006b) found that Cry1Ab toxin was present in adult *Chrysoperla* spp. collected from fields of *Bt* corn (Event 176, Compa CB®, Syngenta). They noted that the presence of Cry protein in the green lacewing adults corresponded with pollen shedding and that no or very little Cry protein was found in adults collected before and after pollen shedding. Indirect studies that have sampled

lacewing populations in *Bt* corn fields vs. non-*Bt* control plots have given mixed results. One study showed no significant difference in population sizes (Bhatti et al. 2005), while in another study (Dively 2005) there were significantly fewer lacewing eggs and larvae in the *Bt* plots compared with plots of the non-*Bt* near-isoline. However, this response was thought to be indirectly due to the lack of insect injury and resultant volatiles that are known to attract adults. Wolfenbarger et al. (2008) concluded from a meta-analysis that *Chrysoperla* is not affected in the field by *Bt* crops. A paper was published recently by Li et al. (2008) that evaluated the impact of Cry1Ab (Event 176) and Cry3Bb1-expressing corn pollen on fitness parameters of the adult Old World *C. carnea*. They concluded that adults of this species are not affected by *Bt* corn pollen. Fitness parameters they studied included adult survival over a 28-day period, pre-oviposition period, fecundity, fertility, and dry weight. They also fed adults artificial diet containing 10 times the Cry1Ab and Cry3Bb1 levels in pollen and found no significant differences in fitness parameters (Li et al. 2008). Cry3Bb1 is targeted for corn rootworm (Baute 2004).

Because adult lacewings are often found in high numbers in corn fields feeding on the pollen (Sheldon & MacLeod 1971, 1974), the *Bt* in the pollen could have an influence on their fitness and population dynamics. To our knowledge, no research has examined the potential effects on fitness of adults of *C. plorabunda* feeding on corn pollen produced by transgenic *Bt* corn. Therefore, the purpose of this study was to determine if there is an impact on longevity, fecundity, and egg viability when *Bt* corn pollen is fed upon by adult *C. plorabunda*. Results from our study may help determine if *Bt* Cry proteins present in corn pollen used as food by these adults may have some influence on their biology that could potentially alter the fitness of individuals and affect their population overall.

## Materials and Methods

**Insects.** This study was conducted in the laboratory from the fall of 2002 through the spring of 2004. *C. plorabunda* cocoons were obtained from Ward's Natural Science (Rochester, NY). According to a company source, the stock culture originated from southern California and was identified as *C. plorabunda*. Upon arrival at our laboratory, the cocoons were placed in a Percival Scientific Series 101 environmental chamber (Perry, IA) that was set at constant 25°C and had a photo cycle of 16:8 h L:D until adult emergence. Throughout the studies, adults were maintained and tested under the same environmental conditions. Adult lacewings were fed pure honey as described by Sheldon and MacLeod (1971) along with one of the corn pollen treatments being assessed for its effects on the insects. Depending on availability, either Giant® brand clover honey or prepackaged grade A honey, marketed by Knott's Berry Farm®, was used.

**Plants.** The corn for this study was raised in a greenhouse under supplemental light provided by 1000 w mercury halide Duraglow® luminaires (General Electric Co., Hendersonville, NC) and temperatures ranging from 22–30°C. Plants were grown individually from seeds in 26-L pots containing standard greenhouse potting soil. Corn plants were routinely watered on alternate days and they received fertilizer (21N-5P-20K) at the rate of 200 ppm of N once a week. Pollen was harvested from plants that approximated the size and appearance of field-grown corn. Tassels were shaken into a paper bag during

mid-morning when most of the pollen in corn is shed for the day (Mason & Tracewski 1982). Undersized plants were not included in the pollen harvesting. To reduce moisture content, the gathered pollen was temporarily stored in desiccators containing Drierite® (W. A. Hammond Co., Xenia, OH) and subsequently transported in desiccators to the laboratory. Temporary storage did not exceed two weeks. Before beginning the feeding studies, the pollen was separated from anthers and fragments of tassel using a fine mesh (0.2 mm) sieve. Microscopic examination showed that pollen had purity in excess of 95%. The pollen from each corn type was placed in a covered glass Petri dish that was stored in its own desiccator with Drierite® to keep the pollen dry until it was fed to the lacewing adults. Pollen desiccators were kept in total darkness in an environmental chamber at 15°C. Storage in desiccators did not exceed three months.

Three varieties of *Bt* corn and their respective non-*Bt* near-isolines were tested. They were Mycogen 2249 (Event 176, Cry1Ab, NatureGard®) and its non-*Bt* near-isoline (Mycogen 2250); Pioneer 38G17 (Monsanto MON810, Cry1Ab, YieldGard®) and its non-*Bt* near-isoline (Pioneer 3893); and Pioneer 34N42 (Dow TC1507, Cry1F, Herculex®) and its non-*Bt* near-isoline (Pioneer 34N43). Cry1F is targeted for several lepidopteran pests, including European corn borer (Baute 2004). The *Bt* corn lines will subsequently be referred to as Event 176, MON810, and TC1507. Each *Bt* event (transgenic corn line) and its non-*Bt* near-isoline were raised concurrently in the greenhouse. The concentration of the Cry protein in pollen has been reported as 1.1–7.1 μg/g for Event 176, 0.09 μg/g for MON810 (Sears et al. 2001), and 0.11–0.17 μg/g for TC1507 (Mendelsohn et al. 2003). Event 176 is not currently registered in the USA (US-EPA 2009).

**Female longevity and fecundity (trial 1).** Event 176 and MON810 (trial 1) investigations were conducted during the fall 2002 where individual females were monitored for both longevity and oviposition. After sex was determined, an adult female and an adult male *C. plorabunda* were placed into a 0.26 liter jar (inverted with lids down). Jar lids were drilled with several small holes for ventilation, and a half-piece of circular filter paper was placed in each jar to serve as substrate for oviposition. Pollen from each *Bt* corn event and each non-*Bt* near-isoline were tested against 30 insect pairs (120 pairs total). Pairs of *C. plorabunda* were left in the jars until oviposition began. At that time, the males were removed, but the females were maintained in individual jars until they died.

Individual *C. plorabunda* females were checked daily and eggs were counted to determine oviposition rates. After the eggs were counted, the filter paper was changed, and the jar lid and inside of the jar were wiped clean to remove all eggs. Tap water was provided daily in the form of a small, saturated cotton plug approximately 2 mm in diameter. Honey and pollen were provided on alternate days to assure a continuous source of carbohydrates and proteins. Water, honey, and pollen were placed on the lid of the inverted jar in small aluminum caps that were removed when the eggs were counted and the jar and lid were cleaned. This process was continued until the death of the female. The number of days of survival was recorded for each individual. Females that did not lay eggs were eliminated from the study, including the recording of longevity. Accidentally killed or escaped individuals were not included in the analysis.

**Female longevity and fecundity (trial 2).** MON810 (trial 2) and TC1507 investigations were conducted during the fall 2003. For this experiment, the group size of both experimental and non-*Bt* near-isoline groups was increased to 35 females each. The rearing methods were the same as described above for the first trial where individual females were monitored collectively for longevity and oviposition in glass jars. Unfortunately, an outbreak of aphids in the greenhouse prevented the MON810 corn from fully reaching the tassel stage and producing enough pollen for completing this experiment. Therefore, data for MON810 were obtained only up to the time when the pollen ran out at day 18. However, adequate pollen was available from TC1507 and its non-*Bt* near-isoline to complete this experiment. Longevity data were collected for females for TC1507 and its non-*Bt* near-isolines in the same manner as in trial 1. Longevity for MON810 trial 2 was not available due to early termination of the test as explained previously. No data were acquired on the longevity of males in these studies as described up to this point.

**Male and female longevity.** To obtain longevity data for males and females fed pollen of MON810 and TC1507, the testing methods were modified. One modification was to use a larger test chamber, which we believed to be an improvement over confining a single individual to a jar. Sexed adults were divided into four groups, one for each of the pollen types. The four treatments consisted of pollen from MON810, its non-*Bt* near-isoline (Pioneer 3893), TC1507, and its non-*Bt* near-isoline (Pioneer 34N43). At the start of the experiment, each group contained 30 males and 30 females, which were maintained in a 12″ × 12″ aluminum mesh collapsible cage obtained from BioQuip® (Gardena, CA). Food and water were placed on the bottom of each cage. Pure honey was put in a depression glass slide, pollen was placed in a small aluminum cap, and water was delivered from a saturated cotton ball.

Each day, fresh water and honey were provided and pollen was replenished so that it was constantly available. A small square of mosquito netting was placed on top of the honey to prevent the lacewings from becoming stuck, but still allowing free access to the food. Cages were changed and cleaned on a four-day rotational cycle. The number of dead insects in each group and the date of death for individual insects were recorded daily.

**Egg viability.** Eggs were sampled during the first female longevity and fecundity experiment (trial 1) for Event 176 and MON810, where females were placed individually in jars. Egg viability was tested by randomly selecting two eggs from each of the first 15 laying females per group. Thus, 30 eggs were tested for each of the *Bt* event and non-*Bt* near isoline treatments. Eggs were placed individually into two-dram shell vials that were closed at the end with cotton and monitored daily for development and eclosion.

**Statistical analysis.** To compare between treatment means in each of the *Bt* and non-*Bt* groups, data for female longevity, total number of eggs produced per female, and number of eggs produced per female per day were analyzed with a *t*-test where a *P*-value of 0.05 or less was considered significant. Fisher's exact test (Fisher 1954) was used to test for differences in egg eclosion for the *Bt* and non-*Bt* pollen treatments. Because data for male and female longevity in cages were not true replicates, *t*-tests were not conducted and only the means and standard errors are reported.

**Table 1. Longevity of adult *Chrysoperla plorabunda* following eclosion when fed corn pollen from three *Bt* events and their respective non-*Bt* near isolines.**

| Treatment | n | Cohort | No. days post-eclosion Mean ± SE | Maximum | $t$ | $P$ |
|---|---|---|---|---|---|---|
| | | | Maintained in jars | | | |
| Event 176 | 29 | Females | 57.2 ± 3.6 | 103 | 3.206 | 0.002 |
| Isoline-176[a] | 29 | Females | 43.7 ± 2.3 | 74 | | |
| MON810 | 24 | Females | 47.9 ± 3.1 | 90 | 0.850 | 0.399 |
| Isoline-MON810[a] | 29 | Females | 44.2 ± 2.8 | 68 | | |
| TC1507 | 32 | Females | 30.2 ± 2.3 | 55 | 1.675 | 0.099 |
| Isoline-TC1507[a] | 35 | Females | 35.6 ± 2.2 | 59 | | |
| | | | Maintained in cages | | | |
| MON810 | 30 | Males | 64.2 ± 4.6 | 109 | | |
| Isoline-MON810[a] | 30 | Males | 60.1 ± 3.7 | 93 | | |
| MON810 | 27 | Females | 61.4 ± 2.1 | 91 | | |
| Isoline-MON810[a] | 26 | Females | 59.0 ± 3.1 | 98 | | |
| MON810 | 57 | M + F[b] | 62.9 ± 2.6 | 109 | | |
| Isoline-MON810[a] | 56 | M + F[b] | 59.6 ± 2.4 | 98 | | |
| TC1507 | 30 | Males | 67.0 ± 4.5 | 103 | | |
| Isoline-TC1507[a] | 29 | Males | 51.4 ± 4.6 | 107 | | |
| TC1507 | 26 | Females | 57.7 ± 3.3 | 107 | | |
| Isoline-TC1507[a] | 26 | Females | 53.3 ± 2.8 | 73 | | |
| TC1507 | 56 | M + F[b] | 62.7 ± 2.9 | 107 | | |
| Isoline-TC1507[a] | 55 | M + F[b] | 52.3 ± 2.7 | 107 | | |

[a]Non-*Bt* near isoline.
[b]Data for males and females (M + F) were combined.

## Results

**Longevity.** Females raised individually in jars lived significantly longer when fed *Bt* pollen from Event 176 compared with females fed pollen from the non-*Bt* near-isoline (Table 1). Maximum longevity of females fed *Bt* pollen was also longer than females fed the non-*Bt* near-isoline pollen. In contrast, no significant affects of *Bt* pollen feeding on female longevity were evident for MON810 or TC1507 compared with their non-*Bt* near-isolines (Table 1).

For the cage experiments, longevity of males and females appeared fairly consistent between MON810 *Bt* pollen and the non-*Bt* near-isoline treatments (Table 1). However, males fed TC1507 *Bt* pollen lived an average of 67.0 days compared with 51.4 days for males fed the TC1507 non-*Bt* near-isoline pollen; however, these data were not analyzed statistically (Table 1).

**Fecundity.** The daily mean egg production by females fed Event 176 pollen was similar to that for females fed the non-*Bt* near-isoline pollen (Fig. 1). Mean

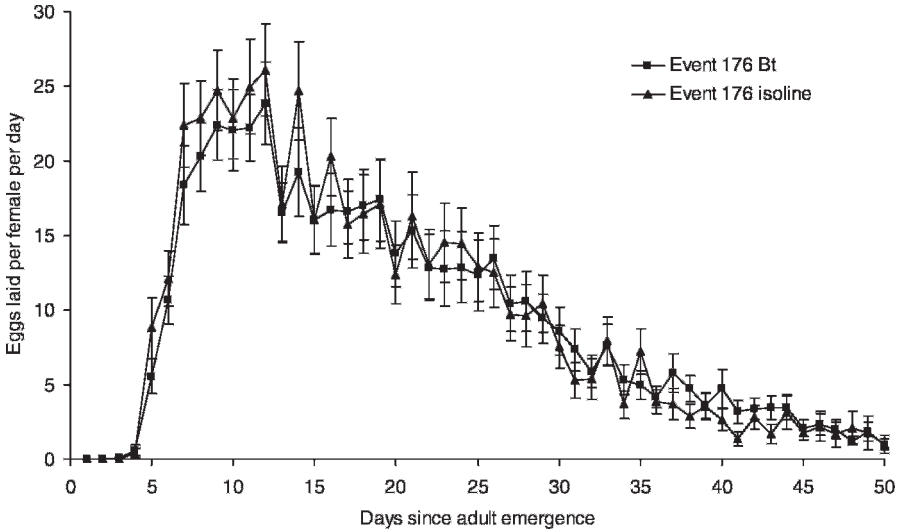


**Fig. 1.** Mean (±SE) eggs laid per day per female for *Chrysoperla plorabunda* when adults were fed pollen from Event 176 *Bt* corn and pollen from its non-*Bt* near-isoline.

number of eggs produced per day per female was not significantly different between those fed Event 176 pollen (10.3) and those fed the non-*Bt* near-isoline pollen (10.7) (Table 2). Also, there was no significant difference in the mean number of total eggs produced per individual for females fed Event 176 pollen (462.0) and those fed the non-*Bt* near-isoline pollen (473.9) (Table 3). The maximum total number of eggs was 818 for one female fed Event 176 pollen and 892 for one fed the non-*Bt* near-isoline pollen.

**Table 2. Comparison of eggs laid per day per female from day 5 following adult eclosion when fed corn pollen from each *Bt* event and its respective non-*Bt* near isoline.**

| Treatment | Days | n | Mean ± SE | *t* | *P* |
|---|---|---|---|---|---|
| Event 176 | 5–50 | 46 | 10.3 ± 1.0 | 0.220 | 0.826 |
| Isoline-176[a] | 5–50 | 46 | 10.7 ± 1.2 | | |
| MON810 (trial 1) | 5–50 | 46 | 8.2 ± 0.7 | 4.052 | 0.001 |
| Isoline-MON810 (trial 1)[a] | 5–50 | 46 | 13.9 ± 1.2 | | |
| MON810 (trial 2) | 5–18[b] | 14 | 17.6 ± 1.0 | 2.149 | 0.041 |
| Isoline-MON810 (trial 2)[a] | 5–18[b] | 14 | 20.9 ± 1.2 | | |
| TC1507 | 5–50 | 46 | 15.7 ± 1.4 | 0.181 | 0.857 |
| Isoline-TC1507[a] | 5–50 | 46 | 15.4 ± 1.0 | | |

[a]Non-*Bt* near isoline.
[b]Experiment not completed due to lack of pollen.

**Table 3. Comparison of total eggs laid per female for the duration of each trial when fed corn pollen from each *Bt* event and its respective non-*Bt* near isoline.**

| Treatment | n | Mean ± SE | *t* | *P* |
|---|---|---|---|---|
| Event 176 | 29 | 462.0 ± 42.9 | 0.189 | 0.851 |
| Isoline-176[a] | 29 | 473.9 ± 46.5 | | |
| MON810 (trial 1) | 23 | 363.8 ± 49.7 | 2.528 | 0.015 |
| Isoline-MON810 (trial 1)[a] | 29 | 573.6 ± 62.4 | | |
| MON810 (trial 2) | 32 | 247.0 ± 37.0 | 2.219 | 0.030 |
| Isoline-MON810 (trial 2)[a] | 33 | 299.0 ± 45.0 | | |
| TC1507 | 32 | 507.9 ± 52.9 | 0.411 | 0.682 |
| Isoline-TC1507[a] | 35 | 536.3 ± 44.7 | | |

[a]Non-*Bt* near isoline.

For females fed MON810 pollen in trial 1, a reduction in the mean number of eggs per female was observed, particularly between days 7 and 27, compared with females fed the non-*Bt* near-isoline pollen (Fig. 2). There was a significant difference in mean number of eggs laid per female per day between the MON810 *Bt* pollen (8.2) and the non-*Bt* near-isoline treatments (13.9) (Table 2). The mean total number of eggs for the MON810 *Bt* treatment for the entire oviposition period was 363.8, which was significantly different from the 573.6 eggs for the non-*Bt* near-isoline treatment (Table 3). The maximum number of eggs from one

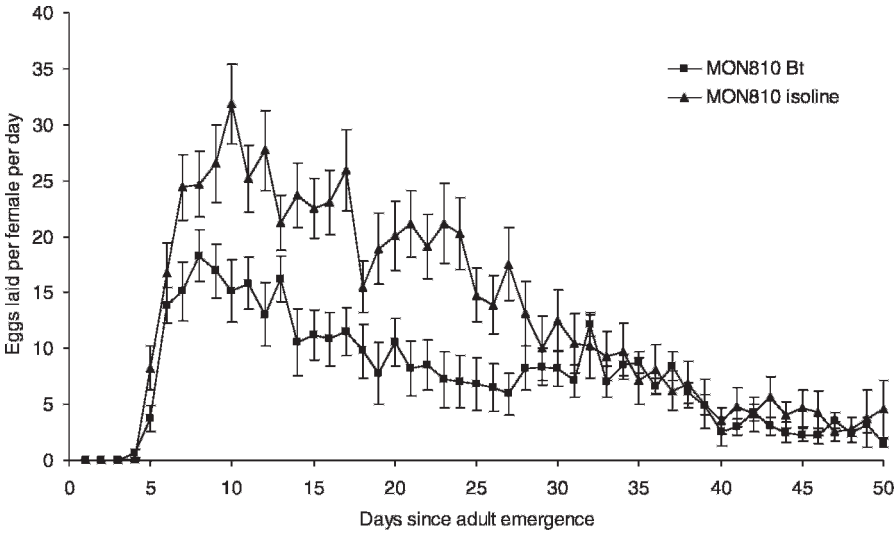


**Fig. 2.** Mean (±SE) eggs laid per day per female for *Chrysoperla plorabunda* when adults were fed pollen from MON810 *Bt* corn and pollen from its non-*Bt* near-isoline for trial 1.

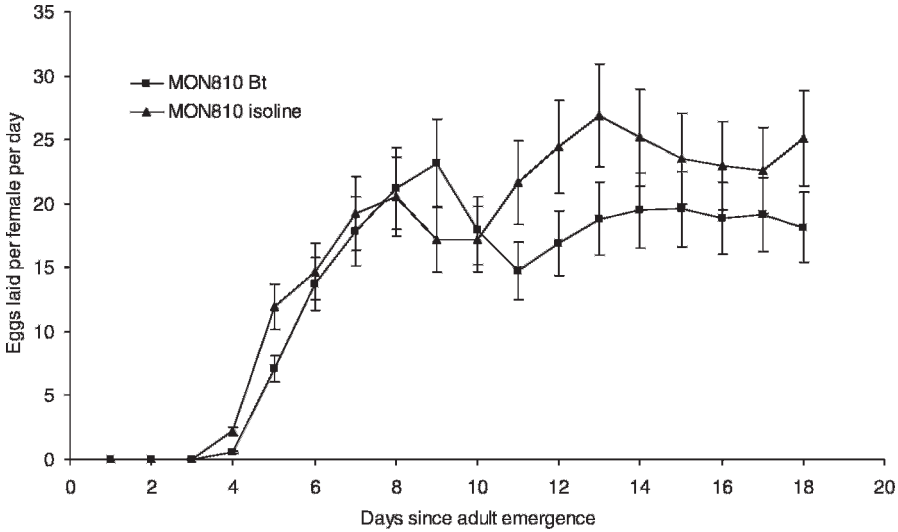


**Fig. 3.** Mean (±SE) eggs laid per day per female for *Chrysoperla plorabunda* when adults were fed pollen from MON810 *Bt* corn and pollen from its non-*Bt* near-isoline for trial 2.

MON810 pollen-fed female in trial 1 was 846 compared with 1321 for a female fed the non-*Bt* near-isoline pollen.

Because pollen for trial 2 ran out after 18 days, this study with MON810 *Bt* was ended early. Even so, females fed MON810 pollen had a noticeably lower oviposition rate from days 10 to 18 than females fed the non-*Bt* near-isoline pollen (Fig. 3). The mean number of eggs produced per day per female from day 5 through day 18 was significantly lower for MON810 (17.6) compared with the non-*Bt* near-isoline pollen (20.9) (Table 2). Total egg production for females fed the MON810 pollen in trial 2 averaged 247.0 eggs during the first 18 days, which was significantly lower than for females fed the non-*Bt* near-isoline pollen (299.0 eggs) (Table 3).

No significant difference in fecundity was detected for females fed TC1507 pollen (15.7 eggs) compared with females fed the non-*Bt* near-isoline pollen (15.4 eggs) (Table 2, Fig. 4). The mean number of total eggs produced by TC1507 pollen-fed females was 507.9 eggs compared with 536.3 eggs for the non-*Bt* near-isoline pollen-fed females (Table 3). Maximum egg production was 1164 for an individual fed TC1507 pollen compared with 1136 for one fed non-*Bt* near-isoline pollen.

**Egg viability.** There were no significant effects on egg development or eclosion rates from females fed pollen from either Event 176 or MON810 in comparison to their respective non-*Bt* near-isolines. Normal appearing larvae emerged from 27 out of 30 of eggs coming from females fed Event 176 pollen compared with 30 out of 30 eggs from females fed the non-*Bt* near-isoline pollen, and this difference was not significant (Fisher's Exact Test, $P = 0.237$). Likewise, 29 out of 30 eggs from females fed MON810 pollen compared with 27 out of 30 eggs from those fed non-*Bt* near-isoline pollen emerged successfully, and this

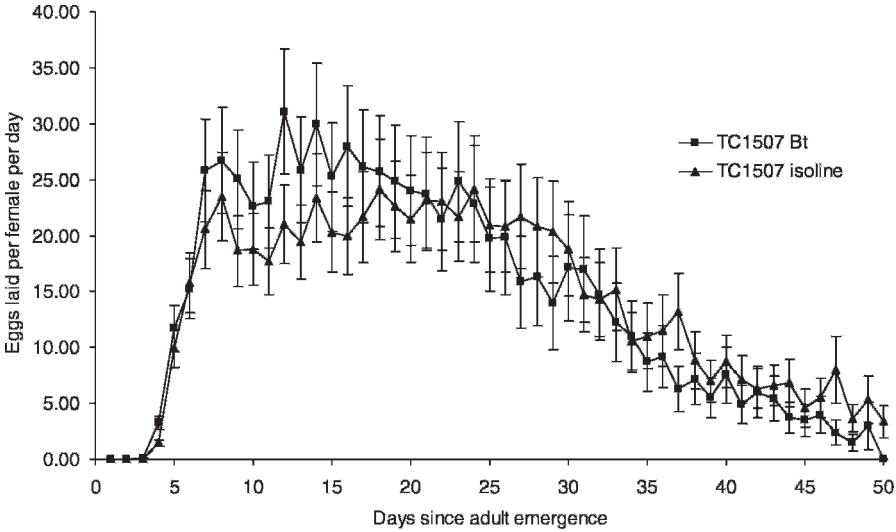


**Fig. 4.** Mean (±SE) eggs laid per day per female for *Chrysoperla plorabunda* when adults were fed pollen from TC1507 *Bt* corn and pollen from its non-*Bt* near-isoline.

difference also was not significant (Fisher's Exact Test, $P = 0.612$). Eggs from females fed pollen from TC1507 also showed no evident reduction in normal development and normal appearing larvae emerged.

## Discussion

The results of our study on longevity differed from a study on the Old-World species *C. carnea* in India (Srinivasan & Sundara Babu 2001), but these studies were designed differently and used *Bt* from totally unrelated sources. Srinivasan & Sundara Babu (2001) reported that *Bt* derived from *Bt* insecticides shortened the life span of *C. carnea*. However, we did not find this to be true for *C. plorabunda*. In fact, the opposite result was shown at a statistically significant level for females fed Event 176 and was indicated as a possibility in the males fed TC1507 in our study. The source of *Bt* for the Indian study (Srinivasan & Sundara Babu 2001) would have contained a wide variety of Cry proteins, being quite different than the single Cry proteins found in the plants of our study. They applied various concentrations of purified *Bt* protein directly into honey as the sole food source, while we fed our adults pollen produced from greenhouse raised corn from three *Bt* events and provided honey as an independent source of carbohydrate without any *Bt* source mixed in. The average longevity of their adults (Srinivasan & Sundara Babu 2001) was approximately one-third of that obtained in our study. This difference in longevity could have been due to a shortage of nutritional protein in the diet fed to adults for their study. As noted previously, pollen (especially corn in agricultural areas) is an important protein source for lacewing adults in Illinois (Sheldon & Macleod 1971, 1974).

Our finding that *C. plorabunda* adults fed *Bt* pollen lived significantly longer in some experiments suggests that there could be a subtle influence by Cry proteins to prolong longevity. Whether an increase in longevity of this predator would be beneficial for pest management or for improving the fitness of *C. plorabunda* is not certain. However, an increase in longevity could be favorable if it increased the overall fecundity by extending the oviposition period or if it increased the mating time for males. In contrast, shorter longevity could result in less egg production by females or reduced reproductive fitness for males.

The MON810 (Cry1Ab) trials resulted in a significant reduction in total egg production by 36.6% (trial 1) and 17.3% (trial 2) compared with its non-*Bt* near-isoline. However, we found no similar significant reduction in fecundity for either Events 176 (Cry1Ab) or TC1507 (Cry1F) pollen. Li et al. (2008) also found no significant difference in fecundity for the Event 176 ('Compa CB') compared with its non-*Bt* near-isoline ('Dracma'). In addition, they found no difference in fecundity for another *Bt* protein, Cry3Bb1, which targets coleopterans rather than lepidopterans. Because both Event 176 and MON810 are based on the Cry1Ab protein, there is an indication that the *Bt* itself may not be responsible for the reduced egg production we observed in MON810 compared with its non-*Bt* near-isoline. Also, since the reported concentration of *Bt* Cry1Ab is more than ten times greater in Event 176 (1.1–7.1 μg/g) than it is in Mon 810 (0.09 μg/g) (Sears et al. 2001), it is questionable whether the reduced fecundity we found for females fed MON810 is due directly to the *Bt* Cry protein. It is not clear why there was a reduced egg production for females fed pollen from this event, but some other factor in the MON810 pollen not associated with *Bt* could be responsible. Other plant-derived compounds, such as agglutinin from *Galanthus nivalis*, have shown a reduction in fecundity of *C. carnea* (Li & Romeis 2009). An alternative possibility is that some factor in the MON810 non-*Bt* near-isoline pollen promoted an increase in egg production compared with the *Bt* pollen.

Our results indicate no impact on eggs when they are produced by females consuming *Bt* corn pollen from the *Bt* events we tested. These eggs appeared to have normal embryonic development and egg eclosion. Li et al. (2008) found no significant effects on egg hatching for Cry1Ab and Cry3Bb1, where hatching rates averaged 82.5% to 87.4%, respectively.

We believe our study is the first to examine fitness criteria for adult *C. plorabunda* that directly fed on pollen derived from *Bt* corn. Previous studies have been on the Old World species, *C carnea*. That we found no reduction in longevity and no impact on egg viability from any of the three *Bt* events we tested corresponds with recent studies on Cry protein that similarly found no negative impact on larvae or adults of *C. carnea* (Romeis et al. 2006, Li et al. 2008). Li et al. (2008) found no negative impact of *Bt* events on survival, fertility, and weight of *C. carnea* adults. Obrist et al. (2006a) found that *Bt* toxin was present in field-collected adults of *C. carnea*, which was associated with the presence of pollen from Event 176 *Bt* field corn. This provides evidence that Cry protein can be present in lacewing adults at least for a period of time. Obrist et al. (2006a) also found *Bt* Cry protein in first instars of *C. carnea* that were fed on prey that had consumed *Bt* from corn tissues. This provides supporting evidence that the Cry proteins can be transferred through herbivores to predators in the food chain. Further evidence for transfer of Cry protein from prey to *C. carnea* larvae is provided by Wei et al. (2008). However, larvae apparently consume some pollen

as a source of supplemental protein, and it is possible that Cry proteins could be obtained this way as well (Patt et al. 2003).

Most of the studies on lacewings in association with Bt crops have been done on larvae. The consensus is that there is minimal or no impact on *Chrysoperla*. In the meta-analysis conducted by Wolfenbarger et al. (2008), they conclude that population numbers of *Chrysoperla* in *Bt* corn and cotton are not affected. However, the findings in our study indicated that reduction in fecundity of *C. plorabunda* adults feeding on MON810 pollen should be noted and investigated further, especially in the field, as it has the potential of having a negative impact on fitness and could reduce the size of the overall population. The MON810 event is currently the most commonly planted in corn acreage among the commercial Cry events planted in the USA.

We suggest that if the recent taxonomic reclassification of *C. carnea* into two sibling species (Brooks 1994) continues to be recognized, it will be important to document the responses that C. *carnea* (Old World) and *C. plorabunda* (New World) populations have to Cry proteins.

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# Assessment of *Chrysoperla plorabunda* Longevity, Fecundity, and Egg Viability When Adults Are Fed Transgenic *Bt* Corn Pollen[1]

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**ABSTRACT** The widespread planting of transgenic corn containing *Bacillus thuringiensis* (*Bt*) Cry endotoxin in its tissues for insect pest control raises the potential for influence on many non-target species including pollen-feeding species of Chrysopidae. This study was conducted to assess fitness parameters associated with longevity, fecundity, and egg viability of adult *Chrysoperla plorabunda* (Fitch) (Neuroptera: Chrysopidae) when fed *Bt* corn pollen. *Bt* products tested with their respective non-*Bt* near-isolines were Event 176 (Cry1Ab), MON810 (Cry1Ab), and TC1507 (Cry1F). Females fed pollen from Event 176 lived significantly longer than those fed pollen from its non-*Bt* near-isoline. Males fed pollen from TC1507 showed a trend for living longer than males fed its non-*Bt* near-isoline pollen, but there was no difference for females regarding this event. The mean number of eggs produced per female per day was significantly less for those fed MON810 pollen compared with females fed pollen from the non-*Bt* near-isoline. Total egg production was significantly less for females fed MON810 pollen vs. females fed pollen from its non-*Bt* near-isoline.

**KEY WORDS** lacewing, *Chrysoperla plorabunda*, *Bacillus thuringiensis*, transgenic corn, non-target insect, fitness

Bioengineered transgenic crops that were developed in the 1990s by incorporating *Bacillus thuringiensis* (*Bt*) Cry protein endotoxin directly into plant tissues to make them highly resistant to pest insects provide very effective pest control (Pedigo & Rice 2009). Incorporation of *Bt* Cry protein into corn through genetic engineering (Koziel et al. 1993) was readily commercialized and *Bt* corn acreage has substantially increased over the past decade. *Bt* corn was planted on 55 million acres in the USA in 2009, representing 63% of the total acreage planted to field corn that year (USDA-NASS 2009). This percentage includes varieties containing *Bt* only (17% of corn acreage) and stacked varieties (46% of corn acreage) containing both *Bt* and herbicide-resistance genes. Corn

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