

**FEASIBILITY OF A NOVEL FEEDING DISRUPTION TEST (FDT) BIOASSAY KIT FOR RAPID RESISTANCE DETECTION OF SUCKING PESTS OF COTTON****J.B. van Kretschmar****L.C. Magalhaes****J. Zhu****R.M. Roe****North Carolina State University, Department of Entomology****Raleigh, NC****A. C. Cohen****Insect Diet & Rearing Research, LLC****Raleigh, NC****Abstract**

With the eradication of the boll weevil, the development of narrow-spectrum insecticides for whitefly control, and the widespread adoption of Bt cotton in the last decade, several species of plant bugs (Miridae) and stink bugs (Pentatomidae) have become major pests of cotton. Reports of tarnished plant bug *Lygus lineolaris* (Palisot de Beauvois) resistance to several classes of cotton insecticides, and of variable insecticide susceptibilities of stink bug species that damage cotton, highlight the need for assays to monitor resistance. Assays in current use have several limitations. Topical assays used for plant bugs and stink bugs are limited to testing insecticides with contact activity. Except in the case of neonicotinoids, vial assays in current use for plant bugs require the addition of plant material as a food source and are limited to contact insecticides. Vial tests for stink bugs are limited to testing insecticides with contact activity. All of the topical and vial assays for these sucking pests rely on a mortality endpoint which is often not easy to read (requires probing the insect and/or discriminating between knockdown versus death) and un-necessarily extends the time to the assay endpoint. Feeding disruption test (FDT) bioassays circumvent these limitations. In FDT assays, insects feed on insecticide in rehydrateable artificial diet mealpads containing a blue indicator dye to mark feeding on the artificial diet. The assay endpoint is the dose-dependent amount of blue feces produced, usually within 24 hours. The objective of the work described in this paper was to determine the feasibility of developing FDT assays for plant bugs and stink bugs. Lab-strain adult tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois), fed different concentrations of  $\beta$ -cyfluthrin and thiamethoxam in rehydrateable NI diet mealpads containing food-grade blue dye in FDT plates showed a dose-response in production of dyed feces for both insecticides. Lab-strain adult brown stink bugs, *Euchistus servus* (Say), fed different concentrations of thiamethoxam in nectar also showed a mortality dose response. Brown stink bugs fed NI diet containing food-grade blue dye produced dyed feces; this approach should also be applicable to resistance monitoring as was the case for the plant bug.

**Introduction**

Several changes during the 1990s resulted in a "low spray environment" in U.S. cotton producing areas (Van Duyn, 2005). These changes included the widespread adoption of Bt cotton to control heliothine lepidoptera, the eradication of the boll weevil, and the development of narrow-spectrum insecticides for control of *Bemisia* whiteflies (Ellsworth, 2008; Van Duyn, 2005). As a result of these changes, there has been a reduction in the use of broad-spectrum insecticides that has led to the emergence of a "sucking bug complex" of plant bug species (Miridae) and stink bug species (Pentatomidae) that has replaced budworms, bollworms, boll weevils, and whiteflies as the major pests of cotton (Leonard, 2008).

The development of resistance to insecticides has been documented since 1914 (IRAC, 2005). Since the 1990s, tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), has been reported to be resistant to several pyrethroids, organophosphates, and cyclodienes used in mid-South cotton (Snodgrass, 2006). Varying susceptibility to pyrethroids and organophosphates of three stink bug species that damage cotton was also shown (Snodgrass et al., 2006; Willrich et al., 2003).

Topical assays and vial tests in current use for resistance-monitoring in plant bugs and stink bugs have several limitations. Topical assays used for plant bugs (Cleveland and Furr, 1979) and stink bugs (Greene et al., 2001) are limited to testing insecticides with contact activity. Except in the case of the nectar feeding assay for plant bugs for neonicotinoid testing developed by Snodgrass and Gore (2007), vial assays in current use for plant bugs (Snodgrass, 1996) require the addition of plant material as a food source and are limited to contact insecticides. Vial tests for stink bugs (Snodgrass et al., 2005) also are limited to testing insecticides with contact activity. All of the topical and vial assays for these sucking pests rely on a mortality endpoint which is often not easy to read (requires probing the insect and/or discriminating between knockdown versus death) and un-necessarily extends the time to the assay endpoint.

Feeding disruption test (FDT) bioassays circumvent the resistance-monitoring limitations of topical and vial assays (Roe et al., 2000, 2002, 2003, 2004, 2005). In FDT assays, insects feed on insecticide in rehydrateable artificial diet mealpads containing a blue indicator dye to mark feeding on the artificial diet. The assay endpoint is the dose-dependent amount of blue feces produced which in some cases is scored in as little as 2 h but for convenience, typically is made in 24 h (Bailey et al., 1998). Additional advantages of an FDT assay for sucking pests would include: a) provides a food-source to the test insect and the assays mimics natural plant-herbivore interactions; b) assay is applicable to insecticides with both contact and ingestion activity; c) feeding-disruption observed in terms of the absence of blue fecal production provides an easy to read endpoint sooner than mortality; c.) rehydrateable mealpads are easily mass-produced and then easily preloaded in the lab with a diagnostic dose of any insecticide; d.) the rehydrateable mealpads can be stored at room temperature for extended periods and require only a small volume of water to hydrate; e) mealpads can be pre-loaded with insecticides or insecticide can be included in the rehydration solution; f) the mealpads are small (proven feasible in 100- $\mu$ l and 200- $\mu$ l volumes) and thus require a minimal amount of insecticide for tests; g) the FDT assay does not require the use of live plant material as green bean; and h) the small mealpad format can be arrayed into easily transported multi-well plates.

The objectives of the current study were to determine the feasibility of developing an FDT assay for plant bugs and stink bugs. To measure contact and ingestion activity of thiamethoxam and the ingestion activity of imidicloprid, Snodgrass and Gore (2007) fed these two neonicotinoids in nectar to tarnished plant bugs and measured mortality. The use of hydrateble artificial diet containing a marker dye and the use of feeding disruption to determine insecticide susceptibility has not been previously considered for plant bugs. In the current study, we fed thiamethoxam and  $\beta$ -cyfluthrin in rehydrateable artificial diet mealpads containing food-grade blue dye to tarnished plant bugs in order to determine whether we could measure a dose response in the production of blue feces. Also, there has been no published effort to feed insecticides to stink bugs in either artificial diet or nectar. In the work described in this paper, we fed artificial diet containing food-grade blue dye to brown stink bugs in order to determine whether they would feed on the diet and produce blue feces. In addition, we fed thiamethoxam in nectar containing an alternative blue dye (trypan blue) to brown stink bugs in order to determine whether we could measure a dose response in production of blue feces.

## **Materials and Methods**

### **Insects**

Newly-emerged tarnished plant bug (TPB) adults, *Lygus lineolaris* (Palisot de Beauvois), were obtained from a New Jersey Department of Agriculture colony (NJ Dept of Agriculture, Division of Plant Industry, Beneficial Insect Lab, Trenton, NJ). The adults were held in waxed cardboard tubs with mesh lids in a growth chamber (Percival Scientific Model I-66NL; Percival Scientific, Inc., Perry, IA) at  $27 \pm 1^\circ$  C, 65% relative humidity, 14 h light: 10 h dark until tested. Insects of either sex were transferred to the test arenas using an aspirator.

Brown stink bug (BSB) adults (*Euchistus servus* (Say)) were obtained from a laboratory colony maintained at North Carolina State University (Roe Lab, Dearstyne Entomology Building, Raleigh, NC). The colony was established in July 2008 from adults collected with a sweep net from a soybean field at Plymouth, NC. The colony is maintained on green beans, peanuts and 10% sucrose in a growth chamber (Percival Scientific Model I-66NL; Percival Scientific, Inc., Perry, IA) at  $27 \pm 1^\circ$  C, 65% relative humidity, 14 h light: 10 h dark cycle. Insects of either sex were transferred to the test arenas using an aspirator and flat forceps.

### **FDT Diet and Nectar**

The artificial NI diet developed for *Lygus lineolaris* and *L. hesperus* by Cohen (2000) was used for the TPB and BSB assays that are described later. Nectar (10% sucrose) was also used in a BSB assay described in more detail below. Blue dye was added to both the NI diet and nectar. Two dyes were used. Food-grade blue dye (blue 1, Betty Crocker Classic Gel Food Colors; Signature Brands, Ocala, FL) was added to NI diet and nectar. The dye content was approximately 0.5 ml of food-grade blue dye per 25 mls of rehydrated NI diet and nectar. An alternative dye, trypan blue (Catalog # T-6146; Sigma Chemical Co., St. Louis, MO) was added to the nectar used in the BSB assay at a concentration of 0.2 mg trypan blue/ml diet.

### **Insecticides**

Baythroid® XL (formulated  $\beta$ -cyfluthrin; Bayer CropScience, Research Triangle Park, NC) and Centric® 40WG (formulated thiamethoxam; Syngenta Crop Protection, Greensboro, NC) were used in the bioassays.

### **Test Arenas and Bioassay for TPB**

The test arena for TPB assays consisted of a 16-well FDT plate (Roe et al., 2000, 2002, 2003, 2004, 2005) in which each well contained a 100- $\mu$ l mealpad of rehydrateable NI diet containing food-grade blue dye as described earlier. Just before the start of an assay, five solutions of Baythroid® XL and Centric® 40WG were prepared in distilled water at 0.1, 1.0, 10.0, 100.0, and 1000.0  $\mu$ g/ml and 0.03, 0.3, 3.0, 30.0, and 300.0  $\mu$ g/ml, respectively. A 65- $\mu$ l volume of insecticide solution was used to rehydrate the mealpads. Control (insecticide-free) mealpads were rehydrated with 65  $\mu$ l of distilled water alone. After rehydration, the mealpads were covered with a Parafilm® disk cut with a # 12 cork borer. TPB adults of either sex were transferred to the 16-well FDT plates (1 insect per well, 8 insects per insecticide dose). After insect transfer, each plate was covered with a vented clear plastic adhesive cover (BIO-CV-16; C-D International, Pitman, NJ). The test plates were held in a growth chamber at  $27 \pm 1^\circ$  C, 65% relative humidity, 14 h light: 10 h dark. Fecal production was assessed every 24 h for 3 d. Fecal production was measured in terms of a visual estimate of the % of the surface area of each FDT plate well covered with blue feces at the time of observation. Means and standard errors for fecal production were calculated using Microsoft software (Microsoft Office Excel® 2003; Microsoft Corporation, Redmond, WA.)

### **Test Arenas and Nectar Bioassay for BSB**

The BSB nectar test arena consisted of a clear 500-ml polypropylene container (Reynolds-16 B13 Del-Pak®; Reynolds Food Packaging, Richmond, VA ) that was covered with cheesecloth to prevent insect escape. Inside the 500-ml container was a 29.6 ml plastic Solo® cup (Solo® graduated soufflé cup, No. P101; Solo Cup Company, Urbana, IL) that held typan blue (0.2 mg/ml) nectar. An absorbent cotton dental wick (Richmond Dental Braided Cotton Roll, No. 210208; Richmond Dental, Charlotte, NC) was inserted through the Solo® cup lid into the nectar reservoir to allow the BSB to feed on the nectar.

Just before the initiation of an assay, thiamethoxam nectar solutions were prepared at 0.01, 0.1, 1.0, 10.0 and 100.0  $\mu$ g/ml. Twenty mls of each thiamethoxam blue-nectar solution was transferred to the Solo® cup in different test arena. The control consisted of 20 mls of nectar without insecticide. Adult BSB were transferred to the test arena with an aspirator. Five BSB were tested per insecticide solution per experiment; the experiment was run twice. The arenas were held in a growth chamber at  $27 \pm 1^\circ$  C, 65% relative humidity, 14 h light: 10 h dark. Fecal production and mortality were assessed every 24 h for 3 d. Fecal production was measured in terms of a visual estimate of the % of the surface area of each test arena covered with blue feces at the time of observation. Mortality was measured in terms of the number of dead insects observed and was expressed in terms of the percent of the total number of insects tested. Insects were judged dead if found unresponsive to prodding with forceps. Means and standard errors were calculated using Microsoft software (Microsoft Office Excel® 2003; Microsoft Corporation, Redmond, WA.)

### **Test Arenas and NI Diet Bioassay for BSB**

The test arena consisted of a white plastic 1-gallon tub (HDPE; Plastic Packaging Corp., W. Springfield, MA). Ten brown stink bugs were added per test arena and then the test arena covered with cheesecloth to prevent the insects

from escaping. NI diet in 8.5 cm x 10.0 cm Parafilm® feeding packets was then rehydrated with distilled water containing blue dye. Two blue dyes were used. The first blue dye used was trypan blue. When trypan blue did not result in blue feces, a second blue dye was used. The second blue dye used was food-grade blue dye. The feeding packets were transferred to the outside surface of the cheese cloth. This allowed the insects to feed through the cheese cloth from the packets of diet. The infested tubs were held in a growth chamber at  $27 \pm 1^\circ \text{C}$ , 65% relative humidity, 14 h light: 10 h dark. The presence or absence of dyed feces on the inside surface of the plastic container was recorded every 24 h for 3 d.

## **Results and Discussion**

### **FDT Ni Diet Assay for TPB**

An example of a tarnished plant bug test arena is presented in Fig. 1. Fecal production by TPB fed  $\beta$ -cyfluthrin and thiamethoxam in rehydrated NI diet FDT plate mealpads containing food-grade blue dye appears in Figs. 2 and 3, respectively. Food-grade blue dye was used because it was found to result in visible blue feces for TPB.



Figure 1. Tarnished plant bug test arena consisting of an FDT (feeding disruption test) plate with 100- $\mu\text{l}$  of rehydrateable, NI artificial mealpads with food-grade blue dye as a feeding marker. Rehydrated mealpads were each covered with a Parafilm disk cut with a #12 cork borer. Once a single insect was placed in each well, the plate was covered with a vented plastic adhesive cover.

Results show that as insecticide concentration increased, fecal production decreased. An insecticide dose response in fecal production was evident during each of the three days studied. In the case of  $\beta$ -cyfluthrin (Fig. 2) at Day 1, fecal production decreased from 34% of the well area covered with dyed feces for the control (insecticide-free) mealpads to 0.4% coverage for the 1000  $\mu\text{g}/\text{ml}$   $\beta$ -cyfluthrin wells. At Day 2, fecal production decreased from 29% coverage of the control well area to 0% (no dyed feces evident) in the 100  $\mu\text{g}/\text{ml}$  wells. At Day 3, fecal production decreased from 28% coverage of the control wells to 0% in the 1000  $\mu\text{g}/\text{ml}$  wells.

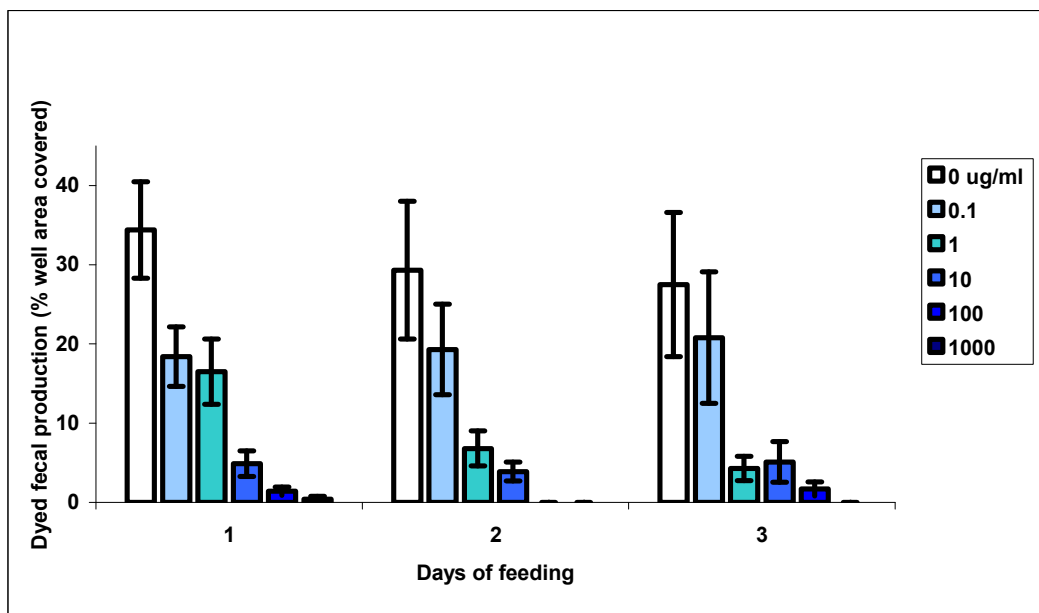


Figure 2. Feeding disruption results for tarnished plant bug adults feeding on blue NI diet mealpads in FDT plates rehydrated with  $\beta$ -cyfluthrin solutions of five concentrations (0.1-1000.0  $\mu\text{g/ml}$ ). Control mealpads were rehydrated with distilled water without insecticide (0  $\mu\text{g/ml}$   $\beta$ -cyfluthrin). Means are for eight insects tested per concentration. Error bars = plus or minus 1 standard error of the mean. Fecal production = percent of FDT plate well surface area covered with blue feces at time of observation. Fecal production was assessed every 24 h for 3 d after the introduction of the insect to the assay.

In the case of thiamethoxam (Fig. 3.), there were no significant differences in the fecal production observed in the control wells when compared to those in which mealpads had been rehydrated with the thiamethoxam concentration of lowest concentration (0.03  $\mu\text{g/ml}$ ). Nevertheless, fecal production decreased with increasing insecticide concentration for each of the three days. At Day 1, fecal production decreased from 39% coverage of the surface area of the control wells with dyed feces to 0% in the 300  $\mu\text{g/ml}$  thiamethoxam wells. At Day 2, fecal production decreased from 49% coverage for the control wells to 0% for the 300  $\mu\text{g/ml}$  thiamethoxam rehydration solution. At Day 3, fecal production decreased from 56% coverage for the control wells to 0% in the 300  $\mu\text{g/ml}$  wells.

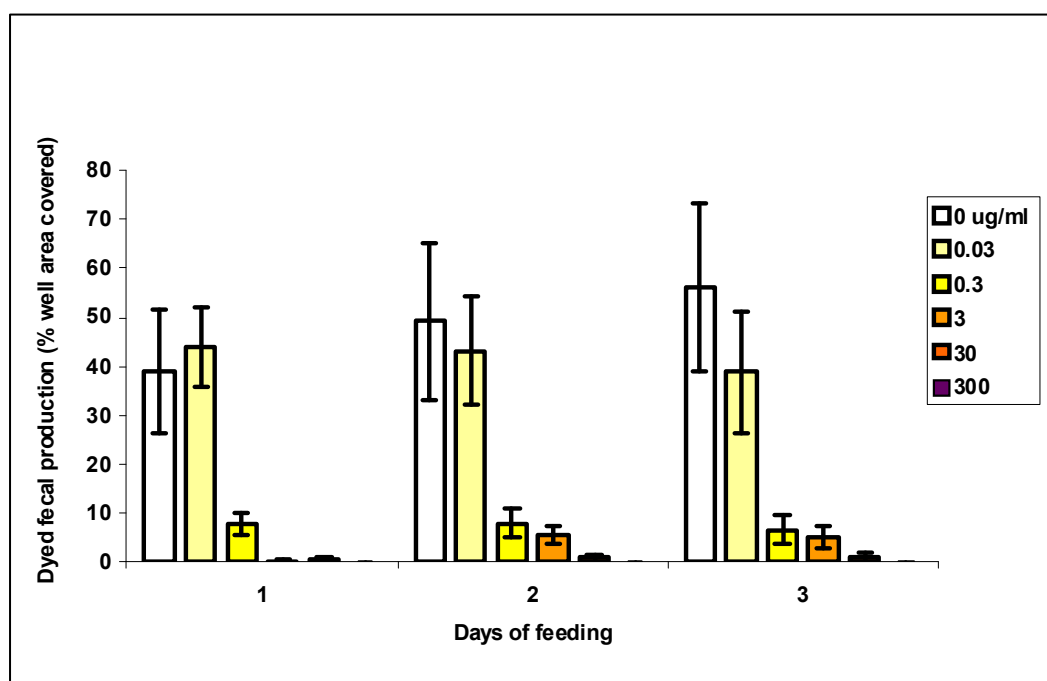


Figure 3. Feeding disruption results for tarnished plant bug adults feeding on blue NI diet mealpads in FDT plates rehydrated with thiamethoxam solutions of five concentrations (0.03-300.0 µg/ml). Control mealpads were rehydrated with distilled water without insecticide (0 µg/ml thiamethoxam). Means are for eight insects tested per concentration. Error bars = plus or minus 1 standard error of the mean. Fecal production = percent of FDT plate well surface area covered with blue feces at time of observation. Fecal production was assessed every 24 h for 3 d after the introduction of the insect to the assay.

The results for these two insecticides demonstrate that production of blue feces can be used as a visual marker in a feeding disruption assay for resistance monitoring using artificial NI blue diet. The insecticide dose-response in fecal production will allow the determination of a diagnostic dose that will distinguish susceptible from resistant TPB. At the diagnostic dose, a resistant insect feeding on rehydrated mealpads will produce blue feces; a susceptible insect will not feed and will not produce blue feces (Roe et al. 2000, 2002, 2003, 2004, 2005). An insecticide dose response in production of blue feces was reported by van Kretschmar et al. (2008) for tobacco budworm (*Heliothis virescens*) moths fed both permethrin and spinosad in nectar containing 0.2 mg/ml trypan blue dye. Current results demonstrate the feasibility of developing an FDT for TPB resistance monitoring.

**FDT Nectar Assay for BSB**

An example of a brown stink bug, trypan blue, nectar feeding test arena is presented in Fig. 4. Mortality results for BSB fed thiamethoxam in nectar containing trypan blue dye is presented in Fig. 5. No control mortality was observed. Trypan blue dye was used because it produced visible blue feces in the lepidopteran FDT (Roe et al. 2000, 2002, 2003, 2004, 2005).



Figure 4. Brown stink bug nectar feeding test arena consisting of a 500-ml polypropylene container holding a 29.6 ml Solo® cup containing nectar with 0.2 mg/ml trypan blue dye. Bugs had access to the nectar from a wick as shown. For each test, the arena contained 5 brown stink bug adults.

Feces produced during the three day assay period were not blue in color. Therefore, blue fecal production could not be visually assessed. Feces from nectar alone was also colorless. Insecticide dose-mortality results for thiamethoxam fed in nectar containing 0.2 mg/ml trypan blue dye (Fig. 5) showed that during the three day assay period, BSB mortality increased with increasing thiamethoxam concentration. Day 1 mortality ranged from 0% mortality for the solutions ranging from 0.0-1.0  $\mu\text{g/ml}$  thiamethoxam to 30% mortality for the 100  $\mu\text{g/ml}$  solution. At Day 2, mortality ranged from 0% mortality for the 0.0-0.1  $\mu\text{g/ml}$  thiamethoxam to 50% mortality for the 10  $\mu\text{g/ml}$  solution; however, from Day 1 to Day 2, there was no change in the mortality for the insects feeding on the 100  $\mu\text{g/ml}$  solutions. At Day 3, mortality increased from 0% mortality for the 0.0  $\mu\text{g/ml}$  control solution to 70% mortality for the 100  $\mu\text{g/ml}$  insecticide solution.

Without a dye marker in the feces, correlation of an insecticide dose-response in fecal production with mortality could not be visually assessed. Prior work had demonstrated a decrease in dyed fecal production for tobacco budworm (*Heliothis virescens*) moths fed increasing concentrations of both permethrin and spinosad in nectar containing trypan blue dye as discussed before (van Kretschmar et al., 2008). The same researchers (van Kretschmar et al., 2008) had shown a plant-sucking Hemipteran, the milkweed bug, *Oncopeltus fasciatus*, produced blue feces when fed nectar containing trypan blue dye for 24 h. The ineffectiveness of trypan blue as a feeding marker for BSB is addressed further in experiments that follow.



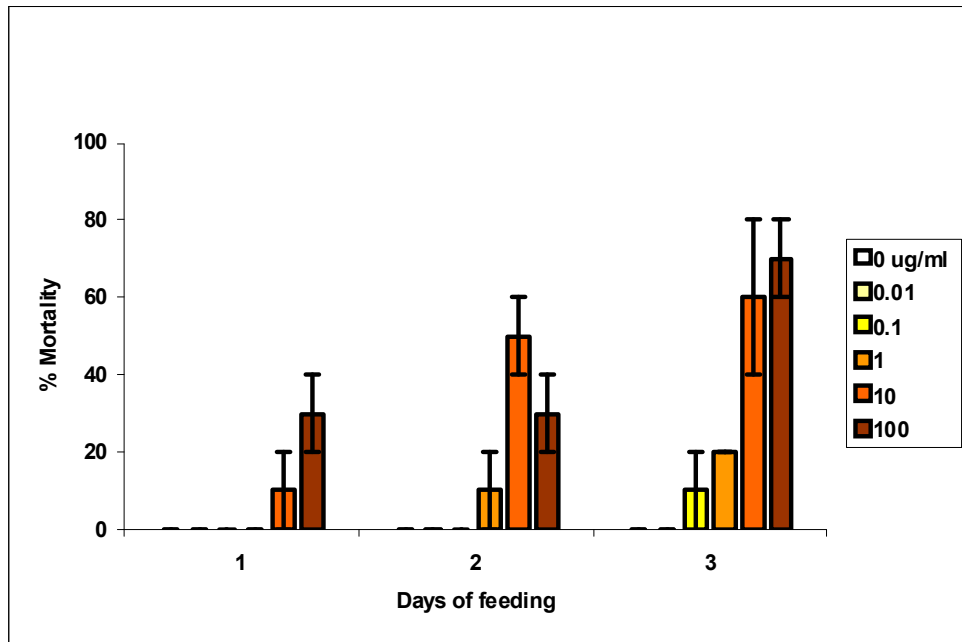


Figure 5. Mortality of brown stink bug adults fed 0.01 - 100.0 µg/ml thiamethoxam in nectar containing 0.2 mg/ml trypan blue dye. Control solution consisted of nectar containing trypan blue dye without insecticide (0.0 µg/ml thiamethoxam). Mortality means are for two experiments of 5 insects tested per thiamethoxam solution per experiment. Error bars = plus or minus 1 standard error of the mean.

#### **FDT NI Assay for BSB**

An example of a brown stink bug NI diet test arena is presented in Fig. 6. Fecal production results for BSB fed NI diet containing trypan blue dye appear in Fig. 7. Fecal production results for BSB fed NI diet containing food grade blue dye are shown in Fig. 8.



Figure 6. Brown stink bug NI diet test arena consisting of a 1 gallon plastic tub containing 10 brown stink bug adults. NI diet containing food-grade blue dye was placed in an 8.5 cm x 10.0 cm Parafilm® feeding packet on top of the cheesecloth lid.



Production of feces by 10 BSB adults fed NI diet containing trypan blue dye for 24 h is presented in Fig. 7. The blue NI diet packet on the right had fed the insects for 24 h and is shown placed in an unused tub for color contrast. The feeding tub on the left of the photo shows the feces produced by the 10 insects during the feeding period. It is evident that the feces did not contain trypan blue dye. These results are consistent with the results reported for BSB adults fed thiamethoxam in nectar containing trypan blue dye (this paper, above). In both cases, BSB failed to produce dyed feces. It is not clear why the trypan blue does not appear in the feces for BSB while this was not an issue for our previous work with the milkweed bug or studies with larval and adult Lepidoptera. We did not examine the use of Trypan blue for the TPB because of the results found for the BSB. The trypan dye is being sequestered during digestion of the BSB and/or the chromophore eliciting the blue color is changed by the process of digestion.



Figure 7. Left, Feces produced by 10 brown stink bugs fed NI diet containing 0.2 mg/ml trypan blue dye during a 24 h test period. The blue feeding packet shown on the right (in an unused tub) had fed insects for 24 h and is shown here after being removed from the feeding tub shown on the left.

Production of feces by 10 BSB adults fed NI diet containing food grade dye blue dye for 24 hours is presented in Figure 8. In contrast to the feces produced by the BSB fed trypan blue dye, the feces produced by BSB fed food-grade dye is blue. These results demonstrate the feasibility of using NI diet with food-grade blue dye to develop a feeding disruption assay with a user-friendly visual marker for feeding disruption for stink bugs. We showed earlier in this paper that this dye also works for TPBs.



Figure 8. Left, Feces produced by 10 brown stink bugs fed NI diet containing food-grade blue dye during a 24 h test period. The blue feeding packet shown on the right (in an unused tub) had fed insects for 24 h and is shown here after being removed from the feeding tub shown on the left.

### Summary

The feasibility of developing an FDT kit for monitoring plant bug and stink bug insecticide resistance was demonstrated. Lab-strain adult tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois), fed different concentrations of  $\beta$ -cyfluthrin and thiamethoxam in rehydrateable NI diet mealpads containing food-grade blue dye in FDT plates showed a dose-response in production of dyed feces for both insecticides. Lab-strain adult brown stink bugs, *Euchistus servus* (Say), fed different concentrations of thiamethoxam in nectar also showed a mortality dose response. BSB fed NI diet containing food-grade blue dye produced dyed feces; this approach should also be applicable to resistance monitoring as was the case for the plant bug.

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