NOVEL ADULT ASSAY TO DETECT INSECTICIDE RESISTANCE OF LEPIDOPTERAN PESTS IN COTTON

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Abstract

Lab-strain tobacco budworm (*Heliothis virescens*) moths were fed different concentrations of permethrin and spinosyn in 10% sucrose containing 0.2 mg/ml trypan blue dye to obtain dose-mortality, dose-nectar-ingestion, and dose-fecal production responses. Percent mortality increased with increasing concentration of permethrin. Ingestion (g of treatment solution per moth) declined with increasing concentration of permethrin. Trends in percent mortality and ingestion for permethrin were consistent with prior results reported for spinosyn (van Kretschmar et al., 2007). Blue-dyed fecal production declined with increasing concentration of both insecticides. These results demonstrate the feasibility of using dyed sucrose solution in an adult-based feeding disruption test to monitor Lepidoptera for development of resistance to chemical insecticides.

Introduction

Current bioassay methods of monitoring insects for development of resistance to chemical insecticides include adult vial tests (Plapp et al., 1987) and larval feeding disruption tests (FDT) (Bailey et al. 1998, 2000, 2001; Roe et al. 2002, 2004, 2005; Khalil et al. 2002; and US Patents Roe et al. 2000, 2003). In the adult vial test, the inside of vials are coated with a chemical toxicant, larvae or moths introduced, and mortality assessed at some fixed time after exposure. Among the disadvantages of the vial test for moths are 1) the assay is only applicable to insecticides with contact activity in the adult stage; 2) the viability of moths in the adult vial test is diminished due to the absence of a nectar source; 3) specialized equipment is required to prepare the vials; and 4) the test is not readily standardized for mass production and possible commercial production.

The larval FDT assay has been developed in a field-kit format. The advantage of the larval FDT kit is that it can be used to monitor for resistance by multiple lepidopteran species to a number of commercial insecticides as well as to Bt proteins. With respect to lepidopteran pests in cotton, an advantage of the larval FDT over the adult vial test is that it can be used to test resistance in caterpillars, the stage which is targeted for control by insecticide treatments. A disadvantage of the larval FDT is the time and effort required to collect eggs and larvae from the field. Among the advantages of an adult FDT assay would be 1) use of lepidopteran adults (moths) that are readily field-collected via light and pheromone traps; 2) the collection of moths allows for monitoring from a greater geographic area than with larvae which are collected directly from individual plants; 3) an assay that is suitable for insecticides with reduced contact activity against both larvae and adults; 4) an assay with the versatility of either feeding disruption or mortality as endpoints; 5) an assay that can be used to monitor single or multiple insects; 6) an assay that supports moth viability during the test by providing nectar (not available for the adult vial test); and 7) an assay format of inherent flexibility for testing multiple chemistries and nectar-feeding species that will be applicable to standardization, mass production and distribution at a minimal cost.

Prior work had demonstrated the feasibility of feeding insecticides un-dyed 10% sucrose (van Kretschmar et al., 2007). The objectives of the current study were to measure the effect of trypan blue dye on ingestion of sucrose solution by moths and the use of this dye as a marker for feeding. In addition, assay feasibility requires that even nectar fed moths collected from the field must feed on nectar in the adult feeding disruption assay. So experiments were conducted to determine if starvation can restore nectar feeding in fed moths. For proof of concept that feeding disruption can be used to monitor insecticide susceptibility, 24-hour nectar ingestion, fecal production and mortality were measured for moths fed, in separate experiments, permethrin or spinosyn in 10% sucrose solution containing 0.2 mg/ml trypan blue dye. To determine proof of concept that the moth adult FDT assay could be used for sucking pests, we examined whether milkweed bugs (*Oncopeltus fasciatus*) would feed on 10% sucrose solution containing 0.2 mg/ml trypan blue dye and produce visually-marked (blue-dyed) feces.

Materials and Methods

Test Arena

An example of the test arena is shown in Figure 1. This was used for laboratory experimentation only and is not expected to be the assay delivery hardware for finalized kits. Each test arena consisted of a white, plastic 1-gallon tub (HDPE; Plastic Packaging Corp., W. Springfield, MA) with a feeding platform that held five 2.5-ml plastic Eppendorf tubes holding 10% aqueous sucrose containing 0.2 mg/ml trypan blue dye (dyed sucrose solution not shown). Feeding platforms consisted of an inverted Styrofoam container (WinCup 8 oz Hot/Cold Food Container, 4" diameter X 2" height) in which holes had been cut via a #6 cork borer. The holes in the Styrofoam container held the Eppendorf tubes. A #2 cork borer was used to cut holes in the lids of the Eppendorf tubes; these holes were large enough to allow moth feeding but small enough to prevent moths from drowning in the sugar solution. The test arena contained five virgin male *H. virescens* moths (0-24 h after emergence) and was covered with clear, plastic food wrap to prevent the moths from escaping and to minimize evaporation of the sucrose solution. Males were used to minimize assay variability associated with reproduction and egg development that occurs in females. Males are also useful for assay since they can be easily collected from the field in pheromone traps.

Fig. 1. Test arena consisting of a 1-gallon plastic tub, a Styrofoam food cup, and five Eppendorf tubes. Test solutions fed to moths were contained by the five Eppendorf tubes.

Insecticides

Test solutions of Tracer® 4 SC (44.2% spinosyns A and D; Dow AgroSciences, Indianapolis, IN) and Pounce® 3.2 EC (38.4% permethrin; FMC Agricultural Products Group, Philadelphia, PA) were prepared in blue-dyed 10% aqueous sucrose containing 0.2 mg/ml trypan blue dye (Catalog # T-6146; Sigma Chemical Co., St. Louis, MO).

Insects

Adult male pupae of *Heliothis virescens* (NCSU Insectary Strain *H. virescens* 02) were obtained from the North Carolina State University insectary. The pupae were held in 1-gallon plastic tubs covered with one layer of cheesecloth and held in a growth chamber (Percival Scientific Model I-66NL; Percival Scientific, Inc., Perry, IA) at 27 ± 1° C, 65% relative humidity, 14 hours light: 10 hours dark for all studies. Eclosed moths (0-1 day after emergence) were immobilized and separated from pupae by chilling at 2° C for 30 minutes. After immobilization, moths were transferred to the test arena.

Feeding Assay

Test arenas containing adult moths were held in the growth chamber at 27 ± 1° C, 65% relative humidity, 14 hours light: 10 hours dark. Mortality and ingestion of the 10% sucrose solution were assessed after a 24 hour incubation. Ingestion was measured as the loss in mass of the feeding platform after 24 hours corrected for evaporation using control arenas without moths and is reported per moth. Mortality was defined as the inability of a moth to right itself from a supine position or to demonstrate coordinated flight. Blue-dyed fecal production was measured by

extracting the test arenas with distilled water and quantifying the extract dye content spectrophotometrically using a trypan blue standard curve. Means and standard errors for mortality, ingestion, and fecal production were calculated using the SAS UNIVARIATE Procedure (SAS, 1999).

Results and Discussion

Effect of Trypan Blue Marker on Nectar Feeding

Ingestion results for dyed sucrose vs. un-dyed sucrose are presented in Figure 2. Ingestion of dyed sucrose declined from 0.21 g solution/ moth at Day 1 to 0.03 g solution/ moth at Day 4. Ingestion of un-dyed sucrose declined from 0.19 g solution/ moth at Day 1 to 0.05 g solution/ moth at Day 4. From Day 4 to Day 7, there was insignificant variation in ingestion of dyed and un-dyed sucrose. These results demonstrate that there was no effect of trypan blue dye at 0.2 mg/ml of nectar on moth feeding, and trypan blue at this concentration can be used as a marker for feeding disruption. It also appears that prior nectar feeding reduces food consumption as might be expected. This could be problematic for an adult FDT test where the insecticide is introduced in nectar, and therefore the prior feeding history could affect insecticide dose.

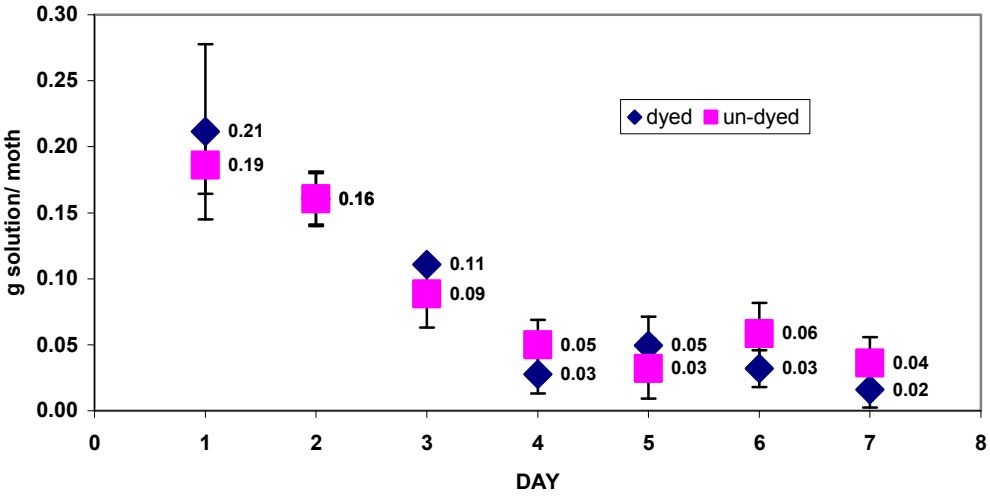


Fig. 2. 24-Hour ingestion of dyed and undyed 10% sucrose solution by male tobacco budworm moths. Dyed sucrose solution contained 0.2 mg/ml trypan blue dye. Error bars = plus or minus 1 standard error of the mean (n=4).

Effect of Starvation on Restoring Nectar Consumption

The results of starvation on moth feeding are shown in Figure 3. The ingestion of moths fed dyed sucrose for the first 24 hours after emergence, Day 1, was 0.21 g solution per moth. After Day 1, the dyed sucrose was withdrawn from the test arena and the moths were starved for 24 hours. At Day 2, dyed sucrose solutions were returned to the test arena, and the moths were allowed to feed again for 24 hours. The Day 3 ingestion was 0.22 g solution per moth which was not significantly different from Day 1. From Day 3 to Day 4, ingestion declined to 0.12 g solution per moth as would be expected based on the results in Figure 2. The results in Figure 3 demonstrate that the appetite of fed moths can be completely restored with 24 hours of starvation; and that the appetite of moths collected from the field regardless of their prior feeding history can be restored by starvation. This will have to be studied further using lab and field collected insects under different conditions of age and levels of prior feeding. This issue of the effect of prior feeding on appetite is important since dosing in the adult FDT assay is *per os,* and nectar feeding is essential for the assay to be feasible. The results in Figure 3 are not too surprising, and the adult FDT assay will likely be feasible. These same issues applied to the larval FDT kit which has now been validated using different instars and at varying times after feeding for field collected caterpillars (Roe, personal communication).

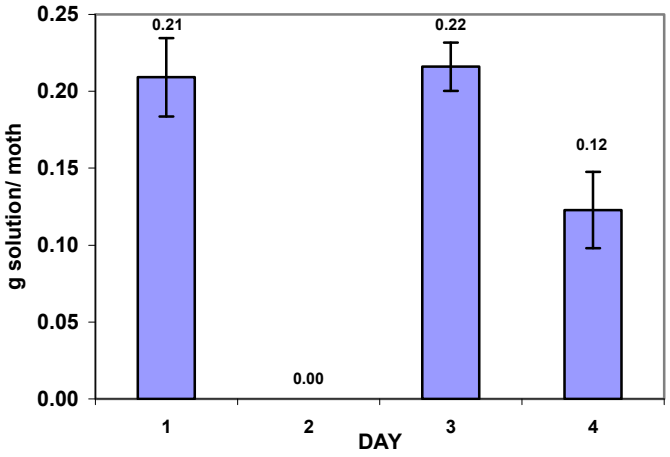


Fig. 3. 24-Hour ingestion of dyed 10% sucrose solution by male tobacco budworm moths. Dyed sucrose solution contained 0.2 mg/ml trypan blue. For the first 24 hours until Day 1, moths were fed. From Day 1 to Day 2, moths were starved; from Day 2 to Day 3, moths were fed. Error bars = plus or minus 1 standard error of the mean (n=4).

Insecticide Dose Mortality Responses Using the Adult FDT Assay

Mortality results 24 hours after feeding moths with permethrin in dyed sucrose are shown in Figure 4. Mortality increased from 0.0% at a permethrin concentration of 1.25 µg/ ml to 100.0% at 20.0 µg/ ml. The trend of increasing mortality with increasing insecticide concentration in nectar has been reported in a prior study with spinosyn (van Kretschmar et al., 2007). These results demonstrate that mortality can be used as an endpoint to monitor insecticide susceptibility using the adult feeding assay.

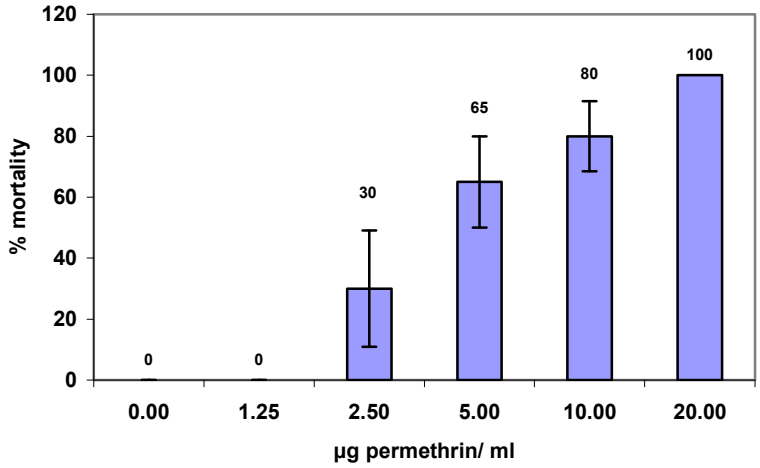
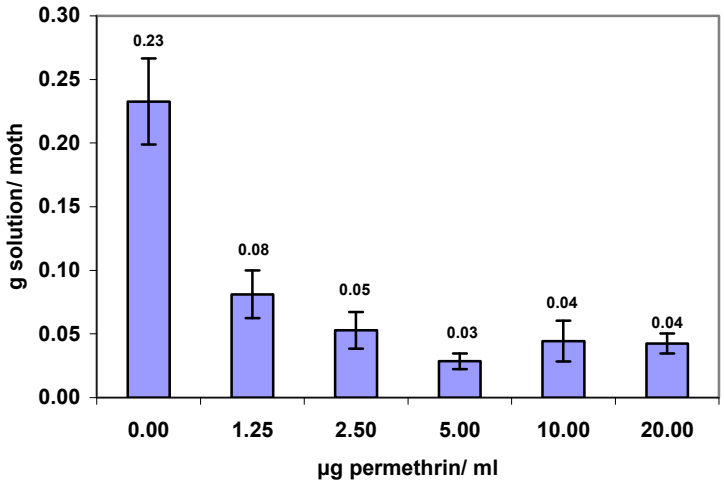


Fig. 4. 24-Hour mortality dose response for male tobacco budworm moths fed permethrin in 10% sucrose solution containing 0.2 mg/ml trypan blue dye. Error bars = plus or minus 1 standard error of the mean (n = 5 for the 0.00 µg permethrin/ ml solution; 4 for the 1.25, 2.50, 5.00, and 10.00 µg permethrin/ml solutions; and 5 for the 20.00 µg permethrin/ ml solution).

Insecticide Dose Ingestion Responses Using the Adult FDT Assay

24-Hour ingestion results for permethrin in dyed sucrose are shown in Figure 5. Ingestion decreased from 0.23 g solution/ moth at a permethrin concentration of 0.00 µg/ ml to 0.08 g solution/ moth at 1.25 µg/ ml. At higher

permethrin concentrations tested, no additional statistically significant reductions in ingestion were noted. The trend of decreasing ingestion with increasing insecticide concentration in the adult FDT assay was shown before for spinosyn (van Kretschmar et al., 2007). These current results for permethrin (Figure 5) and earlier results for spinosyn (van Kretschmar et al., 2007) suggest that using feeding consumption as determined by the amount of nectar consumed can be used to monitor insecticide susceptibility, but in the dose range that caused 100% mortality (as shown in Figure 5 for permethrin) complete cessation of feeding was not possible. It was interesting that a dose of 1.25 micrograms of permethrin per ml produced no morality (Figure 4) but produced a 2.9 fold reduction in feeding (Figure 5). It is not yet clear whether this is the result of repellency or intoxication, and more research will be needed to understand the cause of this reduced feeding. Additional studies will also be needed with permethrin-resistant insects to better understand the relationship between dose and mortality versus reduced feeding to fully evaluate the use of reduced feeding as a valid assay end point.

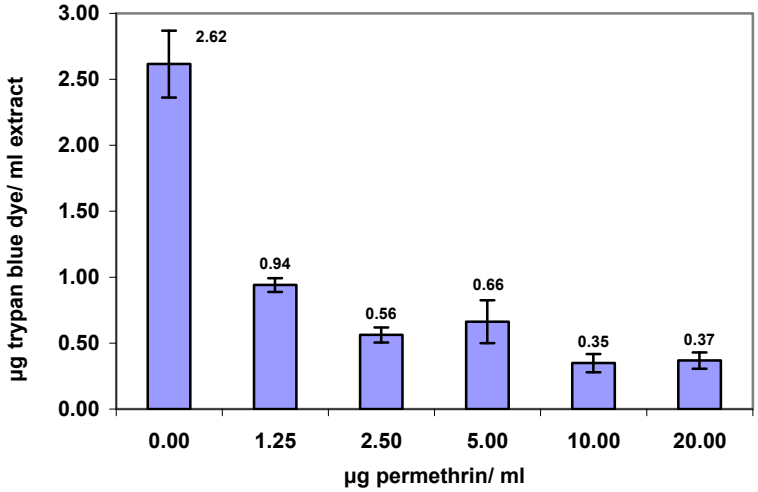


Fig, 5. 24-Hour ingestion dose response for male tobacco budworm moths fed permethrin in 10% sucrose solution containing 0.2 mg/ml trypan blue dye. Error bars = plus or minus 1 standard error of the mean (n = 5 for the 0.00 µg permethrin/ ml solution; 4 for the 1.25, 2.50, 5.00, and 10.00 µg permethrin/ml solutions; and 5 for the 20.00 µg permethrin/ ml solution).

Insecticide Dose Fecal Production Responses Using the Adult FDT Assay

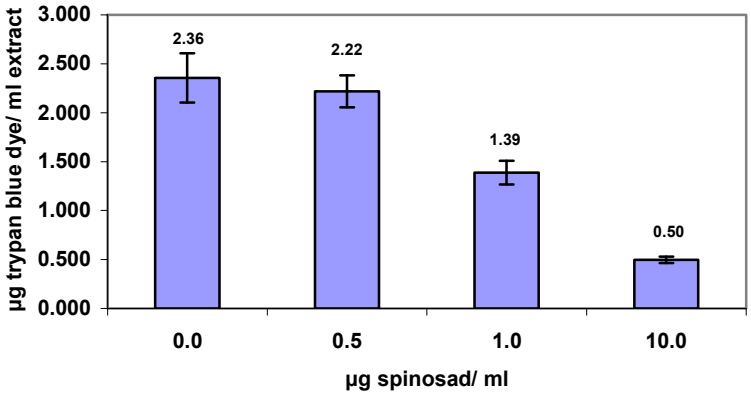
One end point used for the larval FDT assay is fecal production. A blue dye, trypan blue, is added to artificial larval diet to enhance the detection of feces on a white background and to demonstrate feeding on diet containing a diagnostic dose of insecticide. Insects that are susceptible to the pesticide at a diagnostic dose produce minimal blue feces while resistant insects feed normally and produce high levels of blue fecal material. It appears the same approach may be used for an adult FDT assay. As shown earlier (Figure 2), the addition of trypan blue to nectar does not affect moth feeding, and surprisingly, moths feeding on this nectar in the absence of insecticide produce a large amount of feces as shown in Figure 6. The dose fecal production response for permethrin is shown in Figure 7. The amount of dye extracted fell from 2.62 µg trypan blue dye/ ml of extract solution when moths were fed 0.00 µg permethrin/ ml dyed sucrose to 0.94 and 0.56 µg trypan blue dye/ ml of extract solution when moths were fed 1.25 and 2.50 µg permethrin/ ml dyed sucrose, respectively. Higher concentrations of permethrin failed to reduce fecal production further as was also the case for nectar consumption (Figure 5) even though for example at 20 micrograms of permethrin/ml mortality averaged 100% (Figure 4). Again, a dose of 1.25 µg permethrin/ ml dyed sucrose produced no mortality (Figure 4) but reduced fecal production 2.8 fold (Figure 7) and nectar consumption by 2.9 fold (Figure. 5). It appears that both ingestion and fecal production can be used as an assay endpoint for insecticide susceptibility and is capable of detecting sublethal insecticide effects, although as discussed before this needs to be further validated using permethrin resistant strains. Also, further validation of the assay is needed using other insecticides.

Fig. 6. Example of blue-dyed feces produced in 24 hours by male tobacco budworm moths fed 10% sucrose containing 0.2 mg trypan blue dye/ ml.



Fig, 7. 24-Hour dyed-feces production dose response for male tobacco budworm moths fed permethrin in 10% sucrose solution containing 0.2 mg/ml trypan blue dye. Error bars = plus or minus 1 standard error of the mean (n = 5 for the 0.00 µg permethrin/ ml solution; 4 for the 1.25, 2.50, 5.00, and 10.00 µg permethrin/ml solutions; and 5 for the 20.00 µg permethrin/ ml solution).

The amount of trypan blue dye extracted in moth feces from spinosyn test arenas is presented in Figure 8. The amount of dye extracted fell from 2.22 µg trypan blue dye/ ml of extract solution when moths were fed 0.5 µg spinosad/ ml dyed sucrose to 0.50 µg trypan blue dye/ ml of extract solution when moths were fed 10.0 µg spinosad/ ml dyed sucrose. From 0.0 µg spinosad/ ml to 0.5 µg spinosad/ ml, dyed-feces production was invariable. These results demonstrate a dose fecal response where the levels of blue feces decrease with an increasing concentration of spinosyn in the nectar and shows that the adult FDT assay may be applicable to a variety of chemical insecticides.



Fig, 8. Dyed fecal production dose response for male tobacco budworm moths fed spinosad in 10% sucrose solution containing 0.2 mg/ml trypan blue dye. Error bars = plus or minus 1 standard error of the mean (n = 6 for the 0.0 µg spinosad/ ml solution; 4 for the 0.5 and 1.0 µg spinosad/ml solutions; and 5 for the 10.0 µg spinosad/ ml solution).

Application of Adult FDT Tests for Sucking Pests

Sucking pests are increasingly becoming a problem in cotton. Using milkweed bugs as a model, studies were conducted to determine whether this insect would consume nectar containing trypan blue and whether they would produce blue feces like those of moths. Figure 9 shows fecal production of milkweed bugs fed 10% sucrose without (left) and containing (right) 0.2 mg trypan blue dye/ ml. Insects feeding on nectar without the dye produced brown feces while bugs fed trypan blue nectar produced distinguishable fecal deposits that were brown and blue, demonstrating that an adult FDT for sucking pests, such as stink bugs and lygus bugs, might be developed in the future.

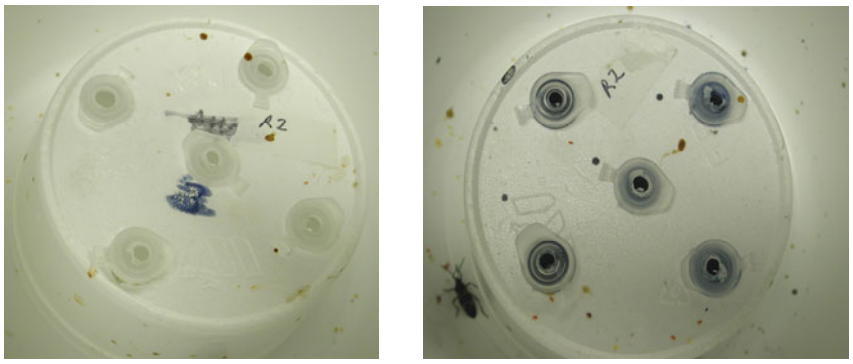


Fig. 9. The photo on the left shows brown feces produced by milkweed bugs fed un-dyed 10% sucrose. The photo on the right shows blue feces produced by milkweed bugs fed dyed 10% sucrose containing 0.2 mg trypan blue dye/ ml.

Summary

The feasibility of developing an adult feeding disruption test (FDT) for moths where the insects are exposed to the insecticide *per os* in 10% sucrose containing trypan blue dye at 0.2 mg/ml was demonstrated. It was shown that trypan blue dye had no effect on nectar consumption and that moth appetite even after feeding *ad libitum* could be restored by starvation. Insecticide dose-response relationships were found for mortality, nectar consumption and blue fecal production demonstrating the use of these as possible assay end points. Additional studies are needed with resistant insects to validate the assay method. Preliminary data are also provided that suggest an adult FDT assay for insecticide resistance can be developed for sucking pests such as stink bugs and plant bugs using the

milkweed bug as a model organism. The adult FDT assay has a number of advantages over that of the adult vial test and the larval FDT assay as discussed and will provide a standardized, easily produced assay architecture which will work with most chemical insecticides and at a minimal cost.

Acknowledgements

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