VALIDATION OF FEEDING DISRUPTION BIOASSAYS FOR SPECIES DIAGNOSIS AND BT-RESISTANCE MONITORING OF BOLLWORM AND TOBACCO BUDWORM FIELD POPULATIONS

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Abstract

Neonates collected as eggs from North Carolina in 1999 were subjected to a 24 h feeding disruption bioassay. The bioassay uses a diagnostic concentration of Bt toxin (0.04 µg MVP per ml diet) to simultaneously monitor for the presence of Bt-resistance in tobacco budworm and for the differentiation of species in the budworm-bollworm complex. The assay proved highly effective at identifying the species of these young larvae, which are not practically distinguishable otherwise. With the implementation of an additional dose of 400 µg MVP per ml diet for bollworm resistance monitoring, no Bt-resistant insects of either species were identified in these field collections. In order to illustrate the predictive value of this technology, 95% confidence intervals were generated for population estimates based on the bioassay response of different sample sizes.

Introduction

Unique IPM challenges are associated with the use of Bt transgenic cotton. Two primary concerns are forestalling the evolution of Bt-resistance in insect populations (Gould, 1988; Tabashnik, 1994; Alstad and Andow, 1995) and differentiating between morphologically similar pest species with varying levels of Bt susceptibility (Stone and Sims, 1993).

Significant laboratory (Tabashnik et al., 1990; Bauer, 1995; Huang et al., 1999) and field data (Tabashnik, 1994; Gould et al., 1997) exist to warrant concern over the loss of Bt insecticidal activity because of the emergence of resistance. The EPA therefore mandates resistance monitoring in transgenic cotton (Matten, 1998) in an attempt to detect such outbreaks as early as possible, should they occur. A common method for monitoring insect resistance is the topical application of insecticides in the adult vial test (Plapp et al., 1987). Adult vial assays are not appropriate for Bt-resistance detection, however, since the mode of entry for Bt intoxication is feeding. The most popular method for detecting resistance to Bt is a larval mortality assay. The disadvantage of this approach is the long assay time (several days). This is impractical for field use and IPM decisions. Since the end point being measured is mortality, the insects can not be used for other diagnostics such as species identification or resistance detection to other insecticides.

Current cotton cultivars expressing the CryIAc Bt toxin are highly toxic to the tobacco budworm, TBW, (*Heliothis virescens*) (Jenkins et al., 1993; Mascarenhas et al., 1994) and less toxic to the bollworm, CBW, (*Helicoverpa zea*) (Stone and Sims, 1993; Lambert et al., 1996). This can result in significant crop damage when CBW populations are high. Also, the need to distinguish between TBW and CBW prior to traditional chemical insecticide applications exists, for example where one species is pyrethroid resistant. Eggs and young larvae of CBW and TBW are indistinguishable in the field by simple observation, requiring an alternative species diagnostic method for making appropriate decisions about pest management. There have been two immunodiagnostic assays commercialized for species diagnosis of eggs (Cibulsky and Ng, 1996; Zeng et al., 1998). While reliable, this approach can be expensive, does not work on larvae and provides no information about resistance.

We recently described a feeding disruption bioassay which could distinguish between laboratory colonies of TBW and CBW while simultaneously diagnosing Bt-resistance (Bailey et al., 1998; Roe et al., 1999; Roe et al., 2000). The endpoint of the assay was the presence or absence of feces when neonates were placed on a diagnostic concentration of the Bt toxin in artificial diet for 24 h. When a blue indicator was added to the diet, larval defecation originating from the bioassay diet could be measured. With this assay system, neonates are collected from the field as eggs.

Laboratory colonies were established in 1999 from field collections of TBW at four sites and CBW at seven sites in the southeastern U.S. (Bailey and Roe, unpublished data). Reliable species diagnosis was demonstrated using a diagnostic concentration of 0.04 µg MVP per ml diet. An easily observable cutoff value of two fecal pellets was established in order to improve the ease of scoring the assay. Bt-susceptible TBW neonates producing ≤ 2 fecal pellets in 24 h were classified as 'non-feeders'. Larvae producing >2 fecal pellets were diagnosed as Bt-susceptible CBW or Bt-resistant insects of either species. The purpose of the current study is to validate this feeding disruption bioassay approach to resistance monitoring and species diagnosis using TBW and CBW larvae collected as eggs from the field.

Materials and Methods

Insects

All insects were routinely reared at $27 \pm 1°$ C with a 14:10 (light:dark) cycle on a standard artificial diet (Burton, 1970). Assayed insects were collected as eggs in the summer of 1999

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in North Carolina. Collections were made on June 22 in Robeson County from tobacco, on August 16 in Beaufort County from non-Bt cotton, and on September 9 and 10 in Edgecombe County on velvet leaf.

Feeding Disruption Bioassay

Our feeding disruption assay technology has been described in detail elsewhere (Bailey et al., 1998; Roe et al., 1999; Roe et al., 2000). The bioassay is conducted with a blue indicator artificial diet containing a diagnostic concentration of insecticide, and the end point measured is the production of blue feces. Trypan Blue (Direct Blue 14; Matheson Coleman and Bell, Norwood, OH) was blended into artificial diet (described earlier) at the rate of 20 mg per 100 ml diet. The blue indicator is used to differentiate feces resulting from the ingestion of assay diet from feces derived from other sources (e.g. egg chorion or cannibalism). The blue indicator diet with MVP (Mycogen, San Diego, CA) was dispensed at the rate of 100 µl per well into 8-well microtitre plate strips (Nalge Nunc Int., Naperville, IL). The wells were sealed with strip caps (Nunc). In order to reduce condensation within the wells, caps were punctured twice with a #3 insect pin.

Assays were conducted on neonates within 24 h of egg hatch using a diagnostic concentration of 0.04 µg MVP per ml diet, with simultaneous no-dose controls. An additional concentration of 400 µg MVP per ml diet was used to monitor CBW Bt-resistance in Edgecombe County larvae.

Morphological Species Determination

Larvae that survived the 24 h feeding disruption bioassay were transferred to artificial diet without MVP and blue dye and reared to adults. Third to last instars were examined under a stereoscope (20-40X) for the presence or absence of microspines on setae tubercles which are characteristic of the TBW and CBW, respectively, as described by Peterson (1948). In our hands, 358 of 359 third to last stadium larvae identified to species by this method were confirmed as the same species as adults. We therefore accepted species confirmations based on either larval or adult morphology. Other species like the tobacco hornworm and loopers could easily be excluded from our analysis as first instars by the caudal horn and walking behavior, respectively.

Mortality

Larval mortality in all experiments is defined by a lack of movement after 10 sec when touched with a blunt probe.

Results and Discussion

Species Diagnosis

The feeding disruption bioassay was successful in identifying TBW and CBW collected as eggs from field populations from North Carolina in 1999. Results for eggs collected from non-Bt cotton in Beaufort County, NC are shown in Fig. 1. No TBW were identified (by morphological examination) in this collection when insects were reared to mature larvae and adults. At a diagnostic concentration of 0.04 µg MVP per ml diet, the majority of neonates produced between 6 to >55 blue fecal pellets per larva in 24 h, with the largest proportion of the population (33.3%) producing 36-45 pellets. 'Feeders' are defined as neonates that produce >2 fecal pellets per larva and in the feeding disruption bioassay for species diagnosis, under current field conditions, would be scored as CBW larvae. Table 1 compares the actual (morphological) species identification to the bioassay results. The feeding disruption assay predicted that 90.5% of the Beaufort larvae were CBW. 'Non-feeders' (producing 0-2 blue fecal pellets) comprised 9.5% of the population and were incorrectly predicted to be TBW (Table 1). This corresponds to an assay error rate of 9.5%.

A 100% TBW population was collected as eggs from tobacco in Robeson County, NC (Fig. 2). Eggs were collected from this source since it was anticipated that these would be primarily TBW and in order to examine the accuracy of the feeding disruption assay for a population at the other extreme from the Beaufort population, which was 100% CBW. All neonates collected as eggs from Robeson County and fed for 24 h on 0.04 µg MPV per ml diet produced 0-2 blue fecal pellets (Fig. 2) and as 'non-feeders' were classified as TBW (Table 1). In comparison to the actual species composition, as determined by morphological examination of mature larvae and adults (Table 1), the feeding disruption assay error rate was 0.0%.

The Edgecombe County collection from velvet leaf yielded a mixed population of TBW and CBW as illustrated in Fig. 3. Of the assayed larvae, 18.7% were predicted by bioassay to be TBW. We found the actual TBW proportion to be 13.5% (Table 1), corresponding to a 38.5% error rate for this species, which is the highest error we observed in these trials. The assay simultaneously predicted 81.3% of the sample population to be CBW; the true CBW proportion was 86.5% (Table 1), representing a 6.0% error rate.

It is worth noting that 23.1% of the Edgecombe TBW were classified as 'feeders' (producing >2 fecal pellets). Although these larvae were incorrectly predicted to be CBW in the feeding disruption assay, only one individual produced >4 fecal pellets (Fig. 3). The remaining larval TBW 'feeders' produced only 3-4 fecal pellets each. In the Robeson County assay (Fig. 2), no TBW larvae produced >2 fecal pellets (Fig. 2). In contrast, the vast majority of CBW produced $\geq$6 fecal pellets in these assays (Figs 1 and 3). These results suggest that larval 'feeders' producing $\geq$6 fecal pellets can be predicted to be CBW with a greater level of confidence.

The data presented on species diagnosis only represents those neonates that did not die during the 24 h feeding disruption

bioassay and which could be identified as TBW or CBW based on morphological characters present in third to last instars and adults. A proportion of the larvae assayed from both the Robeson and Edgecombe samples died before their species could be confirmed (Table 1). If this mortality was due to CryIAc toxicity, there might be concern that susceptible TBW have been unfairly excluded from our analysis. We found no evidence to substantiate this, however. For example, the 10 d mortalities were similar between the 0.04 µg MVP per ml treatment and the control (with no MVP) in both the Robeson and Edgecombe populations. This is especially significant considering that for the Robeson county population, these neonates were 100% TBW, which should be most susceptible to Bt intoxication.

Assay of Larval and Egg Viability

Early (24h) mortality was similar between MVP-treated and control larvae in the Robeson and Edgecombe collections (Table 1). These results demonstrate that in addition to species diagnosis, the feeding disruption assay also can assess 24 h larval viability even in the presence of 0.04 µg of MVP per ml diet. Since the feeding disruption assay is conducted with neonates collected as eggs from the field, it is also possible to assess egg viability. Obviously TBW and CBW pose less of a risk to crop production if they fail to hatch or develop to second instars, regardless of species. Information about egg and larval viability is an important advantage of the feeding disruption assay not found in immunoassays for species diagnosis.

Resistance Monitoring

Previous studies in our laboratory demonstrated that the same diagnostic concentration of MVP used for species diagnosis served to differentiate a Bt-resistant laboratory TBW colony from a Bt-susceptible one (Bailey et al., 1998; Roe et al., 1999). These same studies suggested an additional diagnostic concentration range (400-1200 µg MVP per ml diet) for the monitoring of Bt-resistance in CBW. The use of the feeding disruption bioassay for monitoring the evolution of Bt-resistance in both species under field conditions may eventually prove to be the most useful application of this versatile assay methodology.

Although the potential for the evolution of Bt-resistance is of great concern, there have been no reports of Bt-resistance in field populations of either TBW or CBW; the assumption was made in the current studies that the TBW and CBW larvae collected from North Carolina were not resistant to Bt. In previous studies using multiple TBW field strains collected throughout the SE U.S. in 1998, a small proportion of TBW produced >2 fecal pellets and therefore were classified as 'feeders', but <1% produced >10 fecal pellets at the diagnostic concentration of 0.04 µg MPV per ml diet (Bailey and Roe, unpublished). This suggested that a cut-off of 10 fecal pellets could serve as an initial reference point to monitor for future Bt-resistance in TBW. In the current Robeson County collection (Fig. 2), 100% of the neonates produced 0-2 blue fecal pellets per larva. In this test, the diagnostic concentration simultaneously identified the correct species as TBW (see previous discussion on species diagnosis) and predicted Bt-susceptibility. Bt-susceptibility was also successfully diagnosed in the Edgecombe County collection. This population was a mixture of TBW and CBW (Table 1 and Fig. 3). However, of the TBW, only one larva produced 9 fecal pellets and two larvae produced 4 fecal pellets per larva (Fig. 3), out of a total population of 13 TBW. In this case, the assay again successfully predicted all of the TBW to be Bt-susceptible.

The Edgecombe County collection was predominantly CBW, as determined both morphologically and by bioassay (Table 1). It was previously found (Bailey et al., 1998) that 500 µg MVP per ml diet prevented 'feeding' in a Bt-susceptible CBW laboratory colony, while 200 µg was only partially effective at eliciting the same response. In subsequent studies, 400 µg MVP per ml diet appeared to be a useful concentration for resistance diagnosis in four out of seven CBW field strains collected from the Southeastern U.S. (Bailey and Roe, unpublished data). In the present study, an Edgecombe cohort was bioassayed at 400 µg MVP per ml diet. In this assay designed for monitoring Bt-resistance in CBW, 100% of the neonates produced 0-2 fecal pellets (Fig. 4). Of these, 95.3% died before they could be identified to species based on the morphological characters of the mature larvae and adults. It is apparent that a 24 h exposure to this high concentration was lethal to many of the larvae. Of the 4.7% of the survivors that produced <3 fecal pellets, these were all diagnosed as Bt-susceptible CBW. The percentage CBW determined by morphological examination (with no Bt in the diet) or by the feeding disruption assay (with 0.04 µg MVP per ml diet) was 71.1 and 86.5%, respectively (Table 1). None of these Edgecombe larvae assayed at 400 µg per ml produced >2 fecal pellets. Therefore, all CBW present must be Bt-susceptible. These studies *in toto* show that 0.04 and 400 µg MVP per ml of diet are appropriate diagnostic concentrations for monitoring resistance in TBW and CBW, respectively, using the feeding disruption assay.

Effect of Sample Size on the Accuracy of the Feeding Disruption Assay

The number of randomly sampled insects needed to conduct a successful feeding disruption assay will depend on several factors including the characteristics of the geographical region to be sampled, species compliment and pest density. It is not our intention here to make specific recommendations as to appropriate sample size or collection strategy for the field application of this assay technology. However, it is clear that the confidence placed in population estimates will be increased as sample size increases, as illustrated in Fig. 5. This figure presents 95% confidence intervals for species

predictions based on the feeding disruption bioassay in relation to the true proportion of CBW in the population. Confidence intervals were generated as described in A.15A of Steel et al. (1997) using hypothetical probabilities for 'feeding' by Bt-susceptible TBW and CBW on the 0.04 µg MVP per ml diet diagnostic concentration. The 'feeding' probabilities used to construct these confidence intervals were in the range of those observed in insects from current and previous studies. The errors associated with the bioassay in practice, i.e. 'feeding' TBW and 'non-feeding' CBW, are therefore considered in the calculations of these confidence intervals. If Bt-resistance occurs in future field populations, similar confidence intervals could be generated for resistance detection.

It is apparent that species estimates based on bioassay results are more reliable (the confidence intervals smaller) as the sample size increases (Fig. 5). However, the improvement in the accuracy of the assay for an increase in sample size from 10 to 50 is much greater than an increase in sample size from 50 to 100. Proper integration of these assays into an IPM program will require consideration of the benefits associated with increasing the sample size as compared to the practicality of collecting and assaying large numbers of insects.

The confidence intervals in Fig. 5 illustrate another characteristic of the predictive nature of the bioassay. The feeding disruption assay for species diagnosis is most accurate at true population extremes. As the true population proportion approaches 0 or 100% CBW, the size of the confidence intervals decrease. This variability in the assay accuracy may also affect considerations about sample size.

Summary

The feeding disruption bioassay approach to species determination and Bt-resistance monitoring among heliothine pests is validated here on neonate larvae collected from the field as eggs. The assay provides reliable predictions in 24 h.

Acknowledgments

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References

Alstad, D. N. and D. A. Andow. 1995. Managing the evolution of insect resistance to transgenic plants. Science. 268: 1894-1896.

Bailey, W. D., G. Zhao, L. M. Carter, F. Gould, G. G. Kennedy and R. M. Roe. 1998. Feeding disruption bioassay for species and *Bacillus thuringiensis* resistance diagnosis for *Heliothis virescens* and *Helicoverpa zea* in cotton (Lepidoptera: Noctuidae). Crop Protection. 17(7): 591-598.

Bauer, L. S. 1995. Resistance: a threat to the insecticidal crystal proteins of *Bacillus thuringiensis*. Florida Ent. 78: 414-443.

Burton, R. L. 1970. A low-cost artificial diet for the corn earworm. J. Econ. Entomol. 63: 1969-1970.

Cibulsky, R. J. and S. S. Ng. 1996. Lepton HTK: a diagnostic test kit to improve cotton insect control. Proceedings Beltwide Cotton Conferences. 889-891.

Gould, F. 1988. Genetic engineering, integrated pest management and the evolution of pests. Trends Ecol. Evol. 3: S15-S18.

Gould, F., A. Anderson, A. Jones, D. Sumerford, D. G. Heckel, J. Lopez, S. Micinski, R. Leonard and M. Laster. 1997. Initial frequency of alleles for resistance to *B. thuringiensis* toxins in field populations of *Heliothis virescens*. Proc. Natl. Acad. Sci. USA. 94: 3519-3523.

Huang, F., L. L. Buschman, R. A. Higgins and W. H. McGaughey. 1999. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. Science. 284: 965-970.

Jenkins, J. N., W. L. Parrott, J. C. McCarty Jr., F. E. Callahan, S. A. Berberich and W. R. Deaton. 1993. Growth and survival of *Heliothis virescens* (Lepidoptera: Noctuidae) on transgenic cotton containing a truncated form of the delta endotoxin gene from *Bacillus thuringiensis*. J. Econ. Entomol. 86: 181-185.

Lambert, A. L., J. R. Bradley Jr. and J. W. Van Duyn. 1996. Effects of natural enemy conversation and planting date on the susceptibility of Bt cotton to Helicoverpa zea in North Carolina. Proceedings Beltwide Cotton Conferences. 931-935.

Mascarenhas, V. J., R. G. Luttrell and J. C. Schneider. 1994. Activity of transgenic cotton expressing delta-endotoxin against tobacco budworm. Proceedings Beltwide Cotton Conferences. 1064-1067.

Matten, S. R. 1998. EPA regulation of resistance management for Bt plant-pesticides and conventional pesticides. Res. Pest Man. 10:3-9.

Peterson, A. 1948. An Introduction to Neoarctic species, Part I. Edwards Brothers Incorporated, Ann Arbor, MI.

Plapp, F. W. Jr., G. M. McWhorter and W. E. Vance. 1987. Monitoring for pyrethroid resistance in the tobacco budworm in Texas-1986. Proceedings Beltwide Cotton Conferences. 324-326.

Roe, R. M., W. D. Bailey, G. Zhao, H. P. Young, L. M. Carter, F. Gould, C. E. Sorenson, G. G. Kennedy and J. S. Bacheler. 1999. Assay kit for species and insecticide resistance diagnosis for tobacco budworm and bollworm in cotton. Proceedings Beltwide Cotton Conferences. 926-930.

Roe, R. M., W. D. Bailey, F. Gould and G. G. Kennedy. 2000. Insecticide Resistance Assay. U.S. Patent Application #09/112,274 submitted July 8, 1998.

Steel, R.G.D., J. H. Torrie and D. A. Dickey. 1997. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill, New York. 666pp.

Stone. T. B. and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. J. Econ. Entomol. 86: 989-994.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39: 47-79.

Tabashnik, B. E., N. L. Cushing, N. Finson and M. W. Johnson. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae. J. Econ. Entomol. 83: 1671-1676.

Zeng, F., S. B. Ramaswamy and S. Pruett. 1998. Monoclonal antibodies specific to tobacco budworm and bollworm eggs. Ann. Entomol. Soc. Am. 91(5): 677-684.

Table 1. Percentage mortality, percentage tobacco budworm (TBW) and cotton bollworm (CBW), and 24 h feeding disruption assay results from neonates collected as eggs in North Carolina in the summer of 1999.

County and Dose[a]		n	% Mort. 24h / 10d	ACTUAL[b]		BIOASSAY[c]	
				% TBW	% CBW	% TBW	% CBW
Beaufort	0.04	21	0.0 / 0.0	0.0	100.0	9.5	90.5
	0.00	8	0.0 / 0.0	0.0	100.0	-	-
Robeson	0.04	70	6.8 / 6.8	100.0	0.0	100.0	0.0
	0.00	71	9.9 / 9.9	100.0	0.0	-	-
Edgecombe	400	138	7.2 / 72.5	-	-	-	-
	0.04	143	1.4 / 29.4	13.5	86.5	18.7	81.3
	0.00	113	7.1 / 24.8	28.9	71.1	-	-

[a] Dose is given as µg MVP per ml diet.

[b] Species determinations were made by morphological identification of
mature larvae or adults.

[c] Species predicted by the feeding disruption bioassay, where larvae producing ≤2 fecal pellets are TBW and larvae producing >2 fecal pellets are CBW.

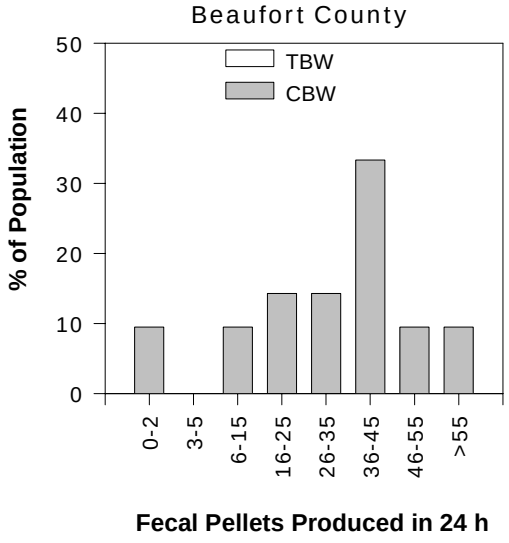

Figure 1. Percent of neonate population producing fecal pellets per larva over 24 h on 0.04 µg MVP per ml diet. Data are taken from 21 neonates hatched from eggs collected from non-Bt cotton in Beaufort County, NC. No TBW eggs were collected from this site. CBW, cotton bollworm; TBW, tobacco budworm.

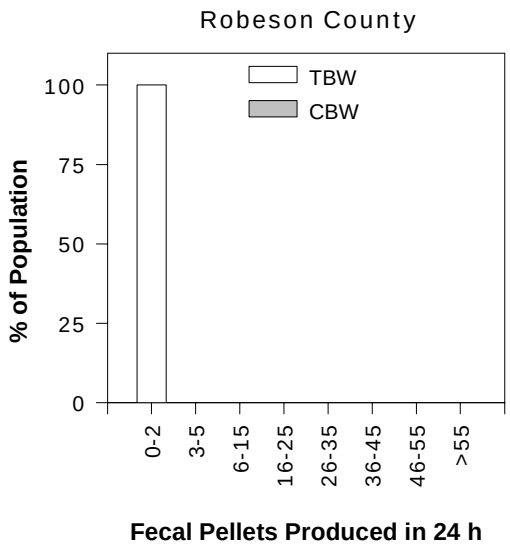

Figure 2. Percent of neonate population producing fecal pellets per larva over 24 h on 0.04 µg MVP per ml diet. Data are taken from 70 neonates hatched from eggs collected from tobacco in Robeson County, NC. No cotton bollworm eggs were collected from this site. CBW, cotton bollworm; TBW, tobacco budworm.

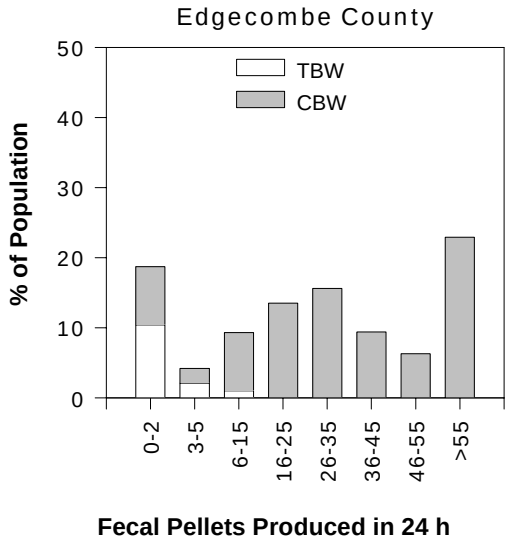

Figure 3. Percent of neonate population producing fecal pellets per larva over 24 h on 0.04 µg MVP per ml diet. Data are taken from 143 neonates hatched from eggs collected from velvet leaf in Edgecombe County, NC. CBW, cotton bollworm; TBW, tobacco budworm.

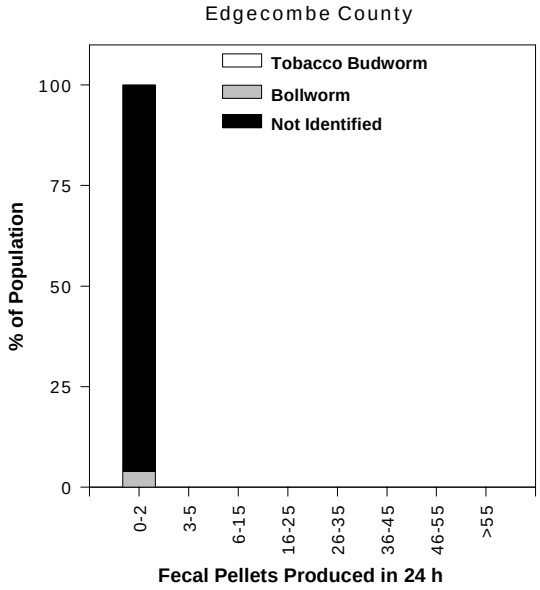

Figure 4. Percentage of neonate population producing fecal pellets per larva over 24 h on 400 µg MVP per ml diet. Data are taken from 138 neonates, hatched from eggs collected from velvet leaf in Edgecombe County, NC. Neonates that failed to develop to mature larvae and adults could not be identified.

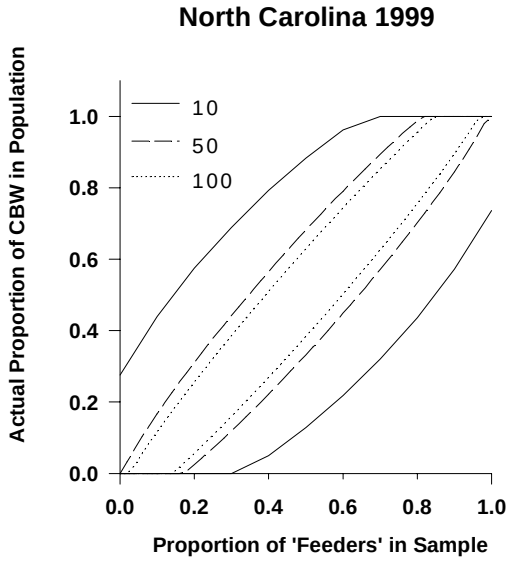

Figure 5. Confidence intervals (95%) generated for the true population proportion of Bt-susceptible CBW corresponding to the proportion of 'feeders' (larvae producing >2 fecal pellets) in a 24 h bioassay at 0.04 µg MVP per ml diet. Confidence bands are generated for random samples of 10, 50 and 100 larvae.