# CATERPILLAR RESISTANCE TO TRANSGENIC COTTON: IMPACT OF INCREASED FEEDING RATES R. Michael Roe Anirudh Dhammi Dominic Reisig North Carolina State University Department of Entomology Raleigh, NC Ryan W. Kurtz Cotton Incorporated Cary, NC

#### Abstract

Currently, caterpillar control in transgenic cotton relies on pyramiding large molecular weight insecticidal proteins and possibly nucleic acids in the future. While there are many benefits to this approach for resistance management, there are also potential weaknesses. If insects can develop a mechanism to prevent all of these biopolymers from reaching the target site the benefits of pyramiding could be reduced. We review the past and present research from our group of the potential impact of increased feeding rates on Bt susceptibility in three insects species, tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*). Increased feeding rates in the former two species produced by increases in temperature result in reduced Bt toxin susceptibility and might explain variations in the effectiveness of Bt cotton in the field. Evidence is also presented of natural variations in feeding rates for the budworm and bollworm before the wide adoption of transgenic cotton and how this is correlated with Bt susceptibility. Finally, laboratory selected Bt resistant budworms and field collected Bt resistant fall armyworms had increased feeding rates than their respective susceptible strains.

## **Introduction**

The adoption of insect-resistant transgenic crops is expanding at double-digit rates (Christou et al., 2006), and this technology has become an essential aspect of integrated pest management for a variety of reasons. These include its level of safety to consumers and the environment, its effectiveness in insect control (especially for caterpillars), and simplification of pest management. The insect protein toxins most often used in transgenic plants are derived from the endotoxin of the bacterium, *Bacillus thuringiensis* (Bt), of which there are several types. All existing evidence indicates that these proteins are safe to the environment, wildlife and human health. Additionally, transgenic plants expressing vegetative insecticidal proteins (VIPs) have been developed and will soon be commercially available in cotton varieties. Nucleic acid-based insect toxins (double stranded RNA, dsRNA) also seem a plausible option in the future for plant expressed insecticides. A common feature of these technologies which theoretically could be significant in terms of resistance is the large molecular weight of these toxins.

The development of resistance to insecticides has been documented since 1914 (IRAC, 2005). Resistance in some species to inorganic insecticides, organic insecticides, Bt sprays, and Bt traits has developed within 2–20 years of being applied to populations of target insects (IRAC, 2005; van Rensburg, 2007; Storer et al., 2010). Probably the best example of this has been with the tobacco budworm, *Heliothis virescens*, in cotton; this insect has developed resistance to a succession of four classes of insecticides since the 1960s (Sparks, 1981; Elzen et al., 1992). To delay the evolution of resistance to insect-resistant transgenic crops, the industry currently relies on pyramiding events expressing two or more insecticidal proteins in the same cultivars. Pyramiding proteins, representing different modes of action, is a proven method for significantly delaying the evolution of resistance (Zhao 2003). Prior to the introduction of pyramided cultivars, it was recognized that genes conferring broad cross resistant may exist in pest populations. However, a broad cross resistance gene would likely provide a fitness advantage on single protein cultivars as well. Given the proven durability of pyramids in the absence of cross resistance, "in short, there is little to lose and potentially a great deal to gain [with pyramiding]" (Roush 1997). The focus of our work is the potential for selecting insect populations with changes in behavior that could reduce access of toxins to the midgut epithelium and result in broad resistance to large molecular weight compounds in general, and the impact of environmental conditions like temperature on susceptibility.

# Rationale Behind Potential Mechanism Affecting Insect Susceptibility to Large Molecular Weight Biopolymers

The insect digestive system is divided into three regions--foregut, midgut and hindgut (Fig. 1, top). Most digestion and

the absorption of nutrients are limited to a relatively short region of the digestive system, the midgut (=ventriculus). As illustrated in Fig. 1, this midgut region is only about a third of the length of the entire digestive tract. Because of this short length, the rate of food movement through the midgut can affect the degree of enzymatic digestion (the rate of conversion of polymers like protein to amino acids) and the rate of absorption of both digested and undigested food. Furthermore, the insect midgut and hindgut is lined with peritrophic membrane (shown in Fig. 1, bottom). This membrane moves along with the food toward the hindgut as the insect eats. The peritrophic membrane structure is optimized to retain larger molecular weight biological polymers like proteins for enzymatic digestion and to permit the much smaller products of digestion (e.g., amino acids) to diffuse across the membrane into the space between the peritrophic membrane and ventricular epithelial cells where they are absorbed into the insect across the midgut brush boarder. Large polymers are impeded from passing through the peritrophic membrane (pore sizes of 0.2 microns in some insects) and being absorbed by the insect. However, this exclusion is not absolute (see review of Jeffers and Roe, 2008).



Figure 1. TOP diagram, basic structure of the insect digestive system; and BOTTOM photo, the midgut epithelium consisting of columnar cells with a brush border lining the gut lumen, muscles on the hemocoel side of the midgut and the peritrophic membrane (arrow) containing food material. Diagram and photo from a model insect to show basic features of the digestive system.

Our hypothesis is that an increased feeding rate, which would increase the rate of movement of food through the insect midgut and also presumably also the rate of movement of the peritrophic membrane, could affect the absorption rate of Bt toxins in the ventricular epithelium. An increased feeding rate might also reduce any enzymatic processing of toxins with alterative effects, i.e., reduced susceptibility if toxin activation is needed or increased susceptibility if toxin degradation is occurring in the midgut lumen. Our group has been interested in examining the rate of food consumption and environmental and population factors that might affect feeding rates on the susceptibility of caterpillars to Bt toxins.

#### **Natural Variations in Feeding Rate**

We examined the feeding rate of three different populations of the tobacco budworm collected directly as eggs from the field in three North Carolina counties (Caberra et al., 2011). The eggs were collected on tobacco not treated with insecticides, were separated from the tobacco tissue in the laboratory, and allowed to hatch in the laboratory. Within 12 h of hatching, the neonates were placed on artificial diet with no Bt. The number of fecal pellets produced over a 24 h period was used as a convenient measure of the rate of food consumption (the more they eat, the more feces are

produced). Among the three populations examined, there was a 2.5-fold variation in feeding rate as measured in terms of fecal production. Whether these differences in feeding rate are a result of heritable traits and/or undetermined environmental factors cannot be determined for certain in these experiments since the insects were collected directly from the field. However, we established tobacco budworm and cotton bollworm (*Helicoverpa zea*) populations in the laboratory from different collection site in the SE US (Bailey et al., 2001). Each population was reared in the laboratory for several generations on artificial diet before L-D probit models were established using fecal production rates as a measure of Bt susceptibility. Natural variations in fecal production rates were found between these populations among each species at low Bt doses, despite being reared for several generations in the laboratory; these results suggest these different feeding rates were inherit in the population and not a result of environmental factors from the field.

### Natural Variations in Feeding Rate Change Bt Susceptibility

In the same experiments just discussed by Bailey et al. (2001) for insects collected from different regions in the SE US and reared in the laboratory for several generations, we also found correlated with low feeding rates, increased Bt toxin susceptibility within each species (budworm and bollworm) and between species, with bollworms demonstrating a much greater feeding rate than budworms and the bollworm having a much lower susceptibility to MVP Bt toxin. Van Kretschmar et al. (2011) from our lab group also found that faster eating bollworms also had a lower susceptibility to Cry1Ac, Cry1Ab and Cry1F than budworms while their feeding rates per neonate and on a caterpillar wet weight bases was higher in bollworms (unpublished).

# Temperature effects on Bt Toxin Susceptibility

There are also methods to artificially change an insect's feeding rate by changing the insect's rearing temperature. It is not uncommon for an increase of 10 degrees C to increase the feeding rate as well as the metabolic rate of an insect by two fold. Van Kretschmar et al. (2013) reported that fecal production at 20 degrees C for neonates of the tobacco budworm was 42% of fecal production at 30 degrees C for a 24 h incubation period on artificial diet. These experiments were repeated with a dose of MVPII in the diet that reduced fecal production at 30 degrees C to 54% of the non-Bt control. Based on the observed effect of temperature alone on the feeding rate, the expected fecal production for neonates of the same strain fed diet containing Bt at 20 degrees C was 15 fecal pellets per larva. However, the actual fecal production rate observed was lower, 60% of the expected rate. The reduced rate of food ingestion and passage rate through the gut may have increased the insect's susceptibility to the Bt toxin. Roe et al. (2014) reported in the same experimental design where the end point measured was not fecal production but mortality, that a 10 degree increase in temperature from 20 degrees C to 30 degrees C reduced percentage mortality by almost one-half in budworms on artificial diet containing both MVPII Bt protein or plant extracts from Bollgard I. In control experiments with no Bt toxin in the diet, temperature change had no effect on mortality in these experiments. These results are interesting because even though the insect is consuming about twice the amount of Bt toxin at the higher temperature, mortality is lower about 2-fold. This reduced toxicity at the higher temperature in these experiments could be the result of the increased feeding rate reducing Bt toxin absorption and/or an increased inactivation of the toxin by gut enzymes. On the other hand and in respect to the latter, if the toxin must be activated by gut enzymes for toxic activity, the effects on mortality by the temperature change could be much larger than what was measured

# Feeding Rates in Laboratory Selected Bt Resistant Tobacco Budworms

Van Kretchmar et al. (2013) also examined the feeding rate of neonates of the YHD2 Bt resistant tobacco budworm strain compared to a Bt susceptible (YDK) strain. These experiments were conducted using adults of similar age reared and allowed to oviposit under identical laboratory conditions and only examined the neonates from eggs produced during a narrow window of peak egg production. The feeding rates for the resistant strain on artificial diet containing no Bt toxin was 37% greater than for the susceptible strain on the same diet. One explanation for these results is that the increased rate of food movement through the gut may be one component of the resistant mechanism for Bt in this strain. The relative importance of the feeding rate versus other possible mechanisms on Bt resistance in the YHD2 strain was not determined.

Our research so far suggests that increased feeding rates in caterpillars can decrease their susceptibility to a variety of different Bt toxins in the tobacco budworm and cotton bollworm determined by fecal production rates and percentage mortality and that increased feeding rates produced by increases in the rearing temperature also can reduced susceptibility by these same measures in the tobacco budworm. Our work also found that budworms selected in the laboratory for resistance to Bt toxin had an increased feeding rate above the parent strain. The work reported here is to determine if these findings also occurred in other caterpillar species, for transgenic cotton with stacked genes for insect

control, and for caterpillars from the field known to be resistant to Bt (they were not selected in the laboratory for resistance).

## **Materials and Methods**

## Insects

Cotton bollworm, *Helicoverpa zea*, eggs were acquired from Benzon research (Benzon Research, Carlisle, PA), and eggs of susceptible and resistant fall armyworms, *Spodoptera frugiperda*, from colonies established by Dr. Dominic Reisig at the Vernon G. James Research and Extension Center (Plymouth, NC). Eggs were incubated at 25 degrees C and newly emerged (in less than 24 h) neonates were used in the experiments described.

### Effect of Artificial Changes in Food Consumption Rate on Susceptibility to Bt Toxin of the Cotton Bollworm

Bioassays with neonates were conducted on artificial diet using hydrateable meal pads as described before by Van Kretschmar et al. (2011, 2013). The meal pads were hydrated with distilled water only, with MVP II Bt toxin in distilled water or with Bollgard II plant extract in distilled water. Insects were allowed to feed for 24 h at 27 degrees C and 14:10 LD after which feeal pellets were counted to establish feeding rates. Food consumption rate was artificially changed in experiments by changes in temperature between 20 to 30 degrees C. Susceptibility of larvae was investigated against MVP II Bt toxin and Bollgard II plant extract using two end points, mortality and fecal pellet production, respectively. For experiments just measuring temperature effects on feeding rate, the plates were hydrated with water only and bioassays conducted in triplicate. For temperature effects for Bollgard II plant extract, meal pads were hydrated with 0.7 µg/ml of Bollgard II plant extract which was prepared as described before from our laboratory (Cabrera et al. 2011); fecal pellets were counted after 24h. Based on the observed effect of temperature alone on the feeding rate, expected fecal production was calculated for diet containing Bollgard II extract at 20° C as described by Kretschmar et al. (2013). Susceptibility of the cotton bollworm was investigated by comparing expected and actual fecal production at 20° C (experiments conducted in triplicate). Mortality experiments at different temperatures were conducted with MVP II toxin. Neonates were reared at 20 and 30 degrees C with a diagnostic dose of 30 µg/ml of MVP II. The mortality was observed after 7 days of feeding. Insects were transferred to new diet every other day. The control was the same experiment without Bt.

# Fecal production of susceptible vs resistant strain of fall armyworm

Assays were conducted using the same hydrateable 16-well FDT plates described before and used above (Van Kretschmar et al. 2011). Neonates of susceptible and resistant fall armyworms were added (one larva per well), incubated for 24 h, and the number of fecal pellets counted (assays replicated five times).

#### Data analysis

A student's t-test was used to test the null hypothesis at alpha = 0.05. Statistical analyses were performed with the use of SAS software (version 9.4, SAS Institute, Cary, N.C.).

#### **Results and Discussion**

## <u>Impact of Reductions in Temperature on Fecal Production in the Cotton Bollworm on Artificial Diet without</u> and with Bt toxin

Previous research (Van Kretschmar et al., 2013) with neonates of the tobacco budworm showed that a 10 degree C reduction in temperature would reduce the feeding rate (measured by the rate of production of fecal pellets over a 24 h period) about 1 half on artificial diet without Bt protein toxin. However, in artificial diet containing a diagnostic dose of Bt toxin, the reduction in fecal production was greater than what would have been predicted by a temperature decrease alone. Fecal production rates have been shown before in detailed log-dose probit modeling to be a measure of Bt protein toxin susceptibility by our group in both the tobacco budworm and cotton bollworm (Bailey et al., 2001). Similar temperature effects were examined for another caterpillar species, the cotton bollworm (Figs. 2-3).



Figure 2. The 24-h average fecal production per cotton bollworm neonate (n = 48) at 30 and 20 degrees C on artificial diet without Bt toxin. Error bar  $\pm$  one standard error of the mean. P<0.0001 (t-test).



Figure 3. The 24-h average fecal production per cotton bollworm neonate (n = 48) fed on artificial diet containing Bollgard II extract at 30 and 20 degrees C.

As expected, a reduction of 10 degrees C (from 30 to 20 degrees C) reduced fecal production in the bollworm to 42.9% of that at 30 degrees C. However, in the presence of a diagnostic dose of an extract of Bollgard II incorporated into the same artificial diet, the fecal production rate observed (Fig. 2) was 53.3% of what was predicted by the impact of the temperature reduction on feeding rate alone. This means, the insects were more susceptible to the Bt toxin at the lower temperature. These results suggest that feeding rates can affect Bt susceptibility not only for budworms (discussed earlier) but also for the cotton bollworm and in this case for Bollgard II, containing pyramided toxins. It is also possible this lower than expected feeding rate could be the result of a lower degradation rate for the toxin at 20 degrees C and/or

feeding rate changes. The work is also of interest because it suggests that changes in temperature in the field could affect caterpillar susceptibility to Bt plants.

#### Impact of Temperature and Feeding Rate on Bollworm Mortality on Bt Artificial Diet

To further demonstrate the impact of temperature and feeding rate changes on Bt susceptibility, we examined the impact of these parameters using mortality as an end point (Figs. 4-5). As was the case previously shown for neonates of the budworm, an increase of 10 degrees C for the cotton bollworm decreased the percentage mortality of a diagnostic dose of the MVPII protein in artificial diet by 29.5% (Fig. 4). This treatment had no impact on mortality in the absence of Bt toxin in the diet (Fig. 5).



Figure 4. Impact of temperature increase on neonate cotton bollworm percentage mortality on artificial diet containing a diagnostic dose of MVPII. The insects were maintained on the test diet for 7 days and then percentage mortality determined. Mortality was defined as no movement when the insect was touched with a blunt probe. Error bar  $\pm$  one standard error of the mean. P = 0.0022 (t-test), n = 48.



Figure 5. Impact of temperature increase on neonate cotton bollworm percentage survival on artificial diet without MVPII toxin. The insects were maintained on the test diet for 7 days and then percentage mortality determined. Mortality was defined as no movement when the insect was touched with a blunt probe. Error bar  $\pm$  one standard error of the mean. P = 0.54 (t-test), n = 30.

# Feeding Rate of a Field Strain of the Fall Armyworm Resistant to Bt Toxin

We showed previously that a Bt toxin resistant strain of the tobacco budworm developed in the laboratory had an increased feeding rate compared to the susceptible parent strain, suggesting a potential mechanism for Bt resistance. To examine this issue further, we examined the feeding rate of a Bt resistant fall armyworm strain collected recently from Herculex field corn in NC and now reared in the laboratory. This was compared to the feeding rate of a susceptible strain, although in this case the susceptible strain was not the origin of the resistant strain. Note that the resistant strain on artificial diet without Bt toxin fed 3.4 times faster than the susceptible strain (Fig. 6).



# Figure 6. Feeding rate of a Bt resistant strain of the fall armyworm as compared to a susceptible strain of the same species. Error bar $\pm$ one standard error of the mean. P < 0.0001 (t-test), n = 80.

Examining the diet surface, there are also physical signs of more dramatic feeding in the resistant strain compared to the susceptible neonates (Fig. 7) as noted by the larger depression on the diet surface. What cannot be determined in these studies is whether our comparative susceptible strain is a reasonable control in these experiments, since the resistant strain was not derived from the susceptible insects. It is possible that we are only observing differences in feeding rates between different populations as was discussed earlier can occur in field populations collected from different sites.



Figure 7. Differences in surface feeding after 24 h between Bt resistant (on left) and Bt susceptible neonates (on right) of the fall armyworm.

#### **Summary**

We present evidence that increased feeding rates in tobacco budworm, cotton bollworm and fall armyworm neonates could be a mechanism for reduced susceptibility and resistance to Bt toxins. We have examined the feeding rates of both a Bt resistant (developed in the laboratory) versus susceptible strain of the tobacco budworm and a Bt resistant

(found in the field in NC) versus susceptible strain of the fall armyworm; in both cases the feeding rate was higher in the resistant strain. Increased insect rearing temperature reduced Bt susceptibility for both susceptible budworms and bollworms suggesting that environmental changes could potentially impact the performance of transgenic cotton in the field relative to caterpillar control. A possible mechanism for a decreased susceptibility to Bt toxins with an increased feeding rate is reduced absorption of the protein toxin by the midgut epithelium. This may be possible due to the peritrophic membrane, designed for the differential retention of large molecular weight biological polymers like protein (and insoluble food material) and the enhanced absorption of the smaller molecular weight products of digestion across the insect midgut epithelium into the hemocoel. This is occurring at the same time as an increased backward movement of the peritrophic membrane due to increased feeding rates. If this model is correct, then the level of activity of any protein toxin or other biological polymer like dsRNA or siRNA could be affected by this mechanism. Furthermore, if this is being used as a mechanism of Bt resistance by insects in the field, the application of transgenic plant technologies could potentially be selecting for caterpillar populations with increased feeding rates. We hope these findings are considered when developing future resistance management plans as well as in current resistance monitoring programs.

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# **References**

Bailey, W.D., C. Brownie, J.S. Bacheler, F. Gould, G.G. Kennedy, C.E. Sorenson and R.M. Roe. 2001. Species diagnosis and *Bacillus thuringiensis* resistance monitoring of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera:Noctuidae) field strains from the southern US using feeding disruption bioassays. J Econ Entomol 94:76-85.

Cabrera, A.R., J. van Kretschmar, J.S. Bacheler, H. Burrack, C.E. Sorenson and R.M. Roe. 2011. Resistance monitoring of *Heliothis virescens* to pyramided cotton varieties with a hydrateable, 'artificial cotton leaf' bioassay. Crop Protection 30: 1196-1201.

Christou, P., T. Capell, A. Kohli, J.A. Gatehouse and A.M.R. Gatehouse. 2006. Recent developments and future prospects in insect pest control in transgenic crops. Trends Plant Sci. 11:302-308.

Elzen, G.W., B.R. Leonard, J.B. Graves, E. Burris and S. Micinski. 1992. Resistance to pyrethroid, carbamate, and organophosphate insecticides in field populations of tobacco budworm (Lepidoptera: Noctuidae) in 1990. J. Econ. Entomol. 85:2064-2072.

Insecticide Resistance Action Committee (IRAC). 2005. Insecticide Resistance: Causes and Action. http://www.irac-online.org.

Jeffers, L.A. and R.M. Roe. 2008. The movement of proteins across the insect and tick digestive system. J. Insect Physiol. 54:319-332.

Roe, R.M., A. Dhammi, L. Ponnusamy, J.B. van Kretschmar and R.W. Kurtz. 2014. Potential new mechanism for insect cross resistance to different protein/dsRNA toxins. 2014 Beltwide Cotton Conferences, New Orleans, LA, January 6-8, 2014. Pp. 878-885.

Roush, R.T. 1997. Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management?. Pest. Sci. 51:328-334.

Sparks, T.C. 1981. Development of insecticide resistance in *Heliothis zea* and *Heliothis virescens* in North America. Bull. Entomol. Soc. Am. 27:186-192.

Storer, N.P., J.M. Babcock, M. Schlenz, T. Meade, G.D. Thompson, J.W. Bing and R.M. Huckaba. 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J.

Econ. Entomol. 103:1031-1038.

van Kretschmar, J.B., W.D. Bailey, C. Arellano, G.D. Thompson, C.L. Sutula and R.M. Roe. 2011. Feeding disruption tests for monitoring the frequency of larval lepidopteran resistance to Cry1Ac, Cry1F, and Cry1Ab. Crop Protection 30:863-870.

van Kretschmar, J.B., A. Dhammi and R.M. Roe. 2013. New mechanism for Bt resistance in caterpillars. Proceedings, Beltwide Cotton Conferences, San Antonio, Texas, January 7-10, 2013. Pages 892-896.

van Rensburg, J.B.J. 2007. First report of field resistance by the stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. S. Afr. J. Plant Soil 27:147-151.

Zhao, J.Z., J. Cao, Y. Li, H.L. Collins, R.T. Roush, E.D. Earle and A.M. Shelton. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nature Biotechnol. 21:1493-1497.