MULTIPLE MECHANISMS FOR CATERPILLAR RESISTANCE TO BT: DON'T FORGET OUR HISTORY

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Abstract

There has been a history of the discovery of new insecticides representing different classes of synthetic chemistry and modes of action (organochlorines, organophosphates, carbamates and pyrethroids) and the evolution of caterpillar resistance. Even with very different chemical classes and modes of action, caterpillars collected from the field demonstrated cross resistance to these pesticides. Conventional thinking would be that selection with a carbamate would slow down resistance to pyrethroids, but in laboratory studies of field collected tobacco budworms, Larvin (a carbamate) treatments increased resistance to cypermethrin (a pyrethroid) faster than treatment with cypermethrin. The use of insect-resistant transgenic crops is wide-spread now and has become an essential aspect of integrated pest management. 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. While there are many benefits to this approach for resistance management, there are also potential weaknesses. If insects possess a mechanism to prevent large molecular weight insecticidal proteins from reaching the target site, the benefits of pyramiding could be reduced. Looking at the lessons from the past, we should not be surprised if caterpillars are eventually able to overcome our best laid plans. Our research has been interested in this possibility and looking at the impact of increase rates of feeding as one method of potential cross tolerance/resistance to protein toxins regardless of their site of action. We have shown before from field populations in the SE US that increased feeding rates were associated with decrease susceptibility to Bt, that decreased temperature and feeding rates increased susceptibility, that a laboratory derived tobacco budworm Bt resistant strain demonstrated increased feeding on non-Bt artificial diet, and that a field collected fall armyworm Bt-resistant strain also demonstrated increased feeding on non-Bt artificial diet. This year we found decreased temperature and feeding rates were again correlated with increased Bt-susceptibility for cotton bollworms for a variety of temperature ranges. We also developed a methodology to group individual bollworm caterpillars (from a single laboratory strain) based on their feeding rates at a fix temperature. When we compared slow versus fast feeders at a single temperature, the latter had a reduced susceptibility to Bt toxin. Although these studies need to be better replicated and correlated with field population variations in feeding rate and Bt susceptibility, *in toto*, it appears caterpillars have at least one mechanism for cross-tolerance or resistance to proteins. More work is needed to better understand this mechanism and its relative potential for impacting the use of Bt cotton for caterpillar control but at least suggests caution is needed in the management of this technology.

Introduction

The development of resistance to insecticides was documented as early as 1914 (IRAC, 2005). Resistance to inorganic insecticides, organic insecticides, Bt sprays, and Bt traits developed within 2–20 years of being introduced into use (IRAC, 2005; van Rensburg, 2007; Storer et al., 2010). As an example, the tobacco budworm, *Heliothis virescens*, in cotton has developed resistance to a succession of four classes of insecticides since the 1960s, the organochlorines, organophosphates, carbamates and pyrethroids (Sparks, 1981; Elzen et al., 1992). This is not unexpected because of the history of insect co-evolution with plants and their genetic variability to adapt to secondary plant compounds aimed to prevent herbivory. The expectation should be with any insecticide use, if the technology is used long enough and on a significant percentage of the population, resistance will develop.

There are situations when the potential for insecticide cross resistance is not so obvious. A good case in point has been the use of organophosphates, carbamates, and pyrethroids where each represents a different class of chemistry and the latter a different site and mode of action. Conventional thinking would be that the alternation of different chemical classes with different modes of action would delay resistance evolution to all of the compounds being used. Rose et al.

(1997) found otherwise. When field collected tobacco budworms were established as a colony in the laboratory and selected either with the carbamate, Larvin, or a pyrethroid, cypermethrin, resistance to cypermethrin occurred faster with Larvin. This was attributed to a new family of P450s that was described at the time, CYP9A1.

The use of insect-resistant transgenic crops is wide-spread now (Christou et al. 2006; James 2014) and 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 for the farmer. 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. 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 et al., 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, "there is little to lose and potentially a great deal to gain [with pyramiding]" (Roush 1997). However, the one common feature of this technology is the focus on Bt derived proteins. If we remember from the past use of synthetic chemistry (discussed earlier), insects were able to develop resistance to insecticides, even when they represented different chemical classes with different modes of action in some cases. Our research has been interested in looking at the impact of increase rates of feeding as one method of potential cross tolerance/resistance to protein toxins regardless of their site of action. One potential mechanism for this cross tolerance/resistance was described by Roe et al. (2015), which was hypothesized as possible for any large molecular weight toxin.

What do we know so far

- There were natural variations in feeding rates between caterpillar populations correlated with differences in Bt susceptibility even before there was Bt cotton.
- Feeding rate can be reduced by reducing temperature; and this reduced feeding rate is correlated with increased susceptibility to Bt for different caterpillar species and for different Bt toxins.
- Budworms selected in lab with Bt have a higher feeding rate than the parent susceptible strain.
- Bt resistant fall armyworms from the field have a higher feeding rate than the susceptible strain.

What do we know so far? There are natural variations in caterpillar feeding rates on artificial diet without Bt (Bailey et al. 2001; Cabrera et al. 2011) between caterpillar species and within a species with higher feeding rates correlated with reduced susceptibility to Bt toxin. Feeding rates can be reduced by a temperature decrease from 30 to 20 ^{o}C on artificial diet without Bt, and correlated with this temperature reduction is an increased susceptibility to different Bt toxins and pyramided cotton for tobacco budworms and cotton bollworms using both fecal production (feeding rate) and/or percentage mortality as end points (Van Kretschmar et al. 2013; Roe et al. 2014, 2015). Furthermore, budworms selected in the laboratory with Bt and which are highly resistant and cross resistant to different Bt toxins have a higher feeding rate than the parental strain on artificial diet without Bt. Moreover, Bt resistant fall army worms, *Spodoptera frugiperda*, collected from the field have a higher feeding rate than the susceptible strain and consume larger meals on artificial diet without Bt (Van Kretschmar et al. 2013; Roe et al. 2014, 2015). This year (i) we examined whether temperature effects on Bt susceptibility may have been an artifact of the temperature range chosen, (ii) we developed a method to partition individual neonates from a single bollworm population into slow and fast feeders, and (iii) we examined the impact of slow versus fast feeding (in the absence of temperature change) on Bt susceptibility.

Materials and Methods

Temperature Effect on Bt Susceptibility

Neonates of a susceptible strain of the cotton bollworm were reared at two different temperatures for different temperature ranges on a diagnostic dose of Bt toxin from MVPII. The appropriate Bt-free controls were also conducted. The impact of temperature change on Bt susceptibility was determined using the rate of fecal production as a measure of Bt susceptibility using our FDT kits described before (Van Kretschmar et al. 2013; Roe et al. 2014, 2015). We have shown before that fecal production rates can be used as a measure of Bt toxicity (Bailey et al. 2001) using FDT.

Development of Slow and Fast Feeding Cotton Bollworm Larvae

Our temperature studies showed that decreased temperature resulted in a reduced feeding rate on artificial diet without Bt and also increased susceptibility to Bt toxin when Bt is added to the diet. However, one concern is that the association with feeding rate and susceptibility is only a correlation. Maybe feeding rate is not the cause of the change in susceptibility, but a decrease in temperature is causing is reducing the inactivation rate of the toxin by proteases or other temperature dependent degradation processes. One approach to examine this question would be to evaluate Bt susceptibility between a population of caterpillars of the same species that were slow and fast feeders.

There are natural variations in feeding rates for cotton bollworms on artificial diet. The mechanism for these differences are not known. We examined the rate of feeding using fecal production with FDT kits for neonates for the first 12 h of larval life and compared these rates to fecal production for the next 24 h. If a correlation could be made between rates for the two time intervals for individual insects, a model could be developed to predict feeding rates for the second 24 h period; this technique could be used to develop slow and fast feeding caterpillar cohorts.

Feeding Rate Impact at the Same Temperature on Bt Susceptibility

The susceptibility of bollworm neonates to MVPII was examined for slow and fast feeders (described for the first time in this paper) and using FDT kits and a diagnostic dose of Bt as described before (Van Kretschmar et al. 2013; Roe et al. 2014, 2015).

Results and Discussion

Impact of Different Temperature Ranges on Feeding Rate and MVPII Susceptibility on Cotton Bollworm Neonates

We previously found that a reduction in temperature from 30 to 20 ^{o}C reduced feeding rates in caterpillars like the tobacco budworm and cotton bollworm on artificial diet without Bt, and this resulted in fecal production at a lower than expected level in the presence of a diagnostic dose of Bt in the diet than what would have been predicted by the impact of temperature alone. It appears from these studies, that the caterpillars were more susceptible to the Bt at the lower versus higher temperature, with the assumption that fecal production is a measure of Bt susceptibility. There is good evidence from our group that this assumption is reasonable based on the probit models developed by Bailey et al. (2001) and our studies where we correlated fecal production rates as a measure of susceptibility to actual mortality for budworms and bollworms (and using different sources of Bt including Bt cotton)(Van Kretschmar et al. 2013; Roe et al. 2014, 2015). In our current studies, we examined for the cotton bollworm whether these temperature effects on Bt susceptibility using MVPII also occurs in a variety of different temperature ranges.

Temperature range 30-20 ^{o}C. Fig. 1 (top) shows the impact of reducing the rearing temperature of cotton bollworm neonates on artificial diet without Bt. Based on this fold reduction in the number of fecal pellets produced in 24 h resulting from the temperature reduction, Fig. 1 (bottom) shows the expected reduction in fecal production in the presence of a diagnostic dose of MVPII in the diet and the actual number of fecal pellets produced at 20 ^{o}C. The actual level of fecal production was 63% of that expected, suggesting an increased susceptibility to the Bt toxin. These results are typical of what we have presented before (Van Kretschmar et al. 2013; Roe et al. 2014, 2015).

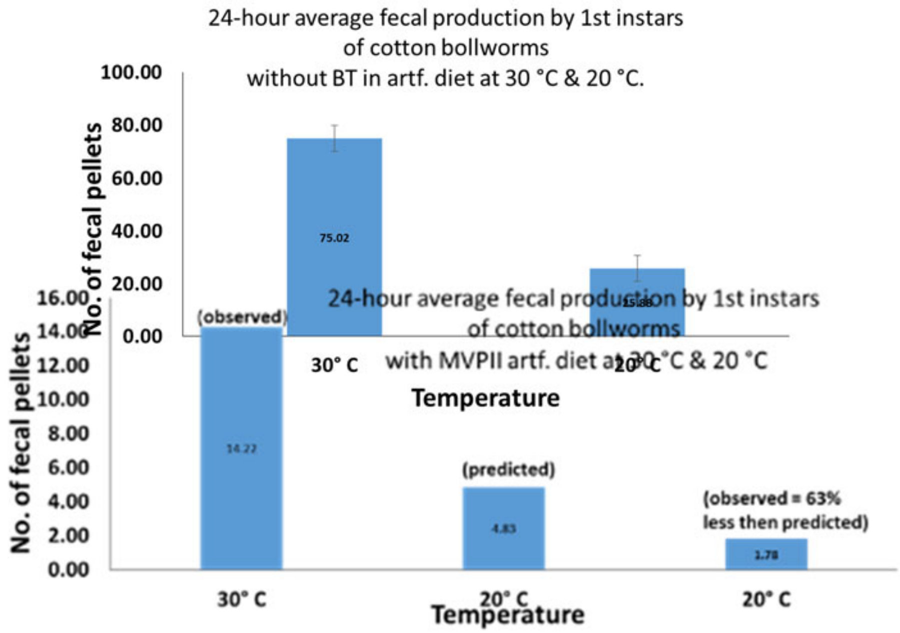
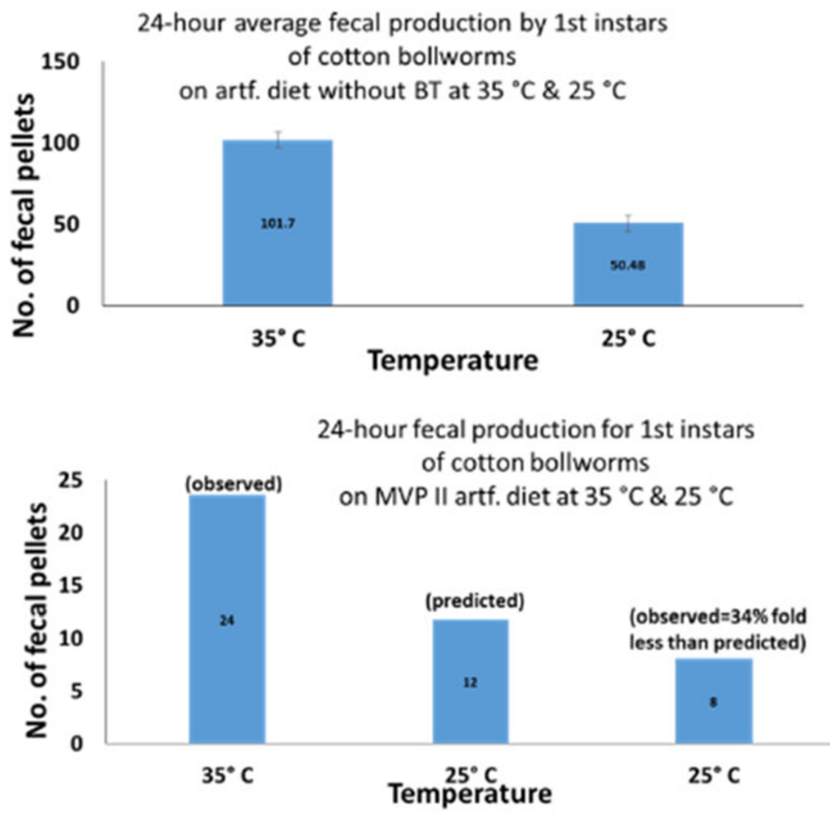


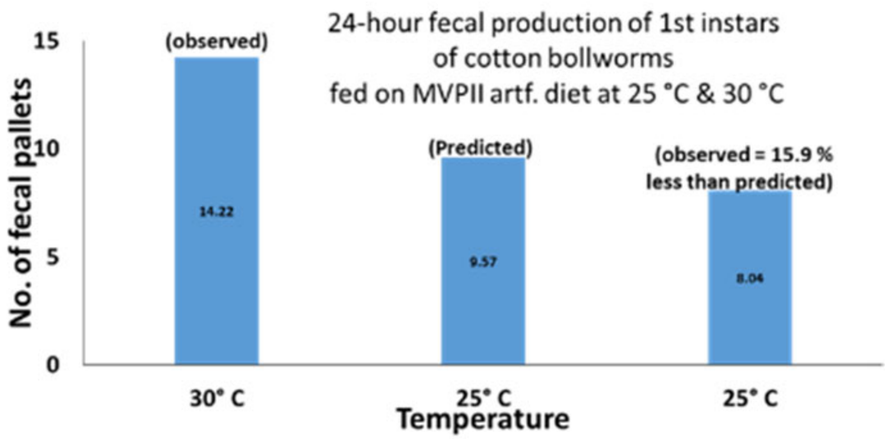
Figure 1. Impact of a change in temperature from 30 to 20 °C on fecal production after 24 h for cotton bollworm neonates on artificial diet without (top) and with a diagnostic dose of MVPII in the meal pad (bottom). The predicted value (bottom) is calculated based on the change in feeding rate without MVPII (top). The error bars (top) are $\pm$1 SEM.

Figure 2. Impact of temperature change from 35 to 25 °C on fecal production after 24 h for cotton bollworm neonates



on artificial diet without (top) and with a diagnostic dose of MVPII in the meal pad (bottom). The predicted value (bottom) is calculated based on the change in feeding rate without MVPII (top). The error bars (top) are $\pm$1 SEM.

Temperature range 35-25 ºC. Similar results were also obtained in the temperature range 35-25 ºC (Fig. 2). The temperature reduction lowered fecal production in artificial diet without Bt (Fig. 2, top) and in the presence of a diagnostic dose of MVPII in the diet, fecal production was 34% lower than the expected (Fig. 2, bottom).



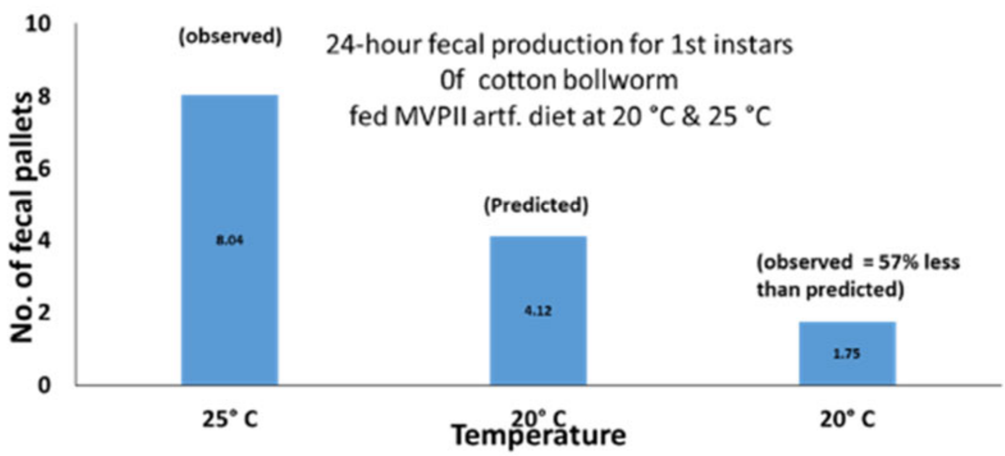


Figure 3. Impact of a change in temperature from 30 to 25 (top) and from 25 to 20 (bottom) ºC on fecal production after 24 h for cotton bollworm neonates on artificial diet with a diagnostic dose of MVPII in the meal pad. The predicted value is calculated based on the change in feeding rate without MVPII in the diet (data not shown for brevity).

Temperature ranges 30-25 and 25-20 ºC.

Lower than predicted fecal production rates were also found at 30-25 and 25-20 ºC, 15.9 and 57% of the expected (Fig. 3; top and bottom, respectively). The temperature studies *in toto* suggest show that the impact of temperature on what appears to be changes in Bt susceptibility is not specific to the temperature range from 30 to 20 ºC reported earlier by our group (Van Kretschmar et al. 2013; Roe et al. 2014, 2015).

Development of Methodology to Partition Individual Bollworm Neonates Based on Their Feeding Rate

When we compared fecal production rates for the first 12 h after hatching to fecal production in the subsequent next 24 h on artificial diet without Bt toxin for individual caterpillars, we found that feeding during the first temporal segment was predictive of the second (Fig. 4, top). The data were variable, but this allowed us to use the first 12 h of feeding to sort neonates into groups of low and high feeders (Fig. 4, middle and bottom). This provided for the first time the ability to examine the effect of feeding rate on Bt susceptibility in the same bollworm population (in a laboratory strain to our knowledge never selected with Bt) in the absence of a temperature change.

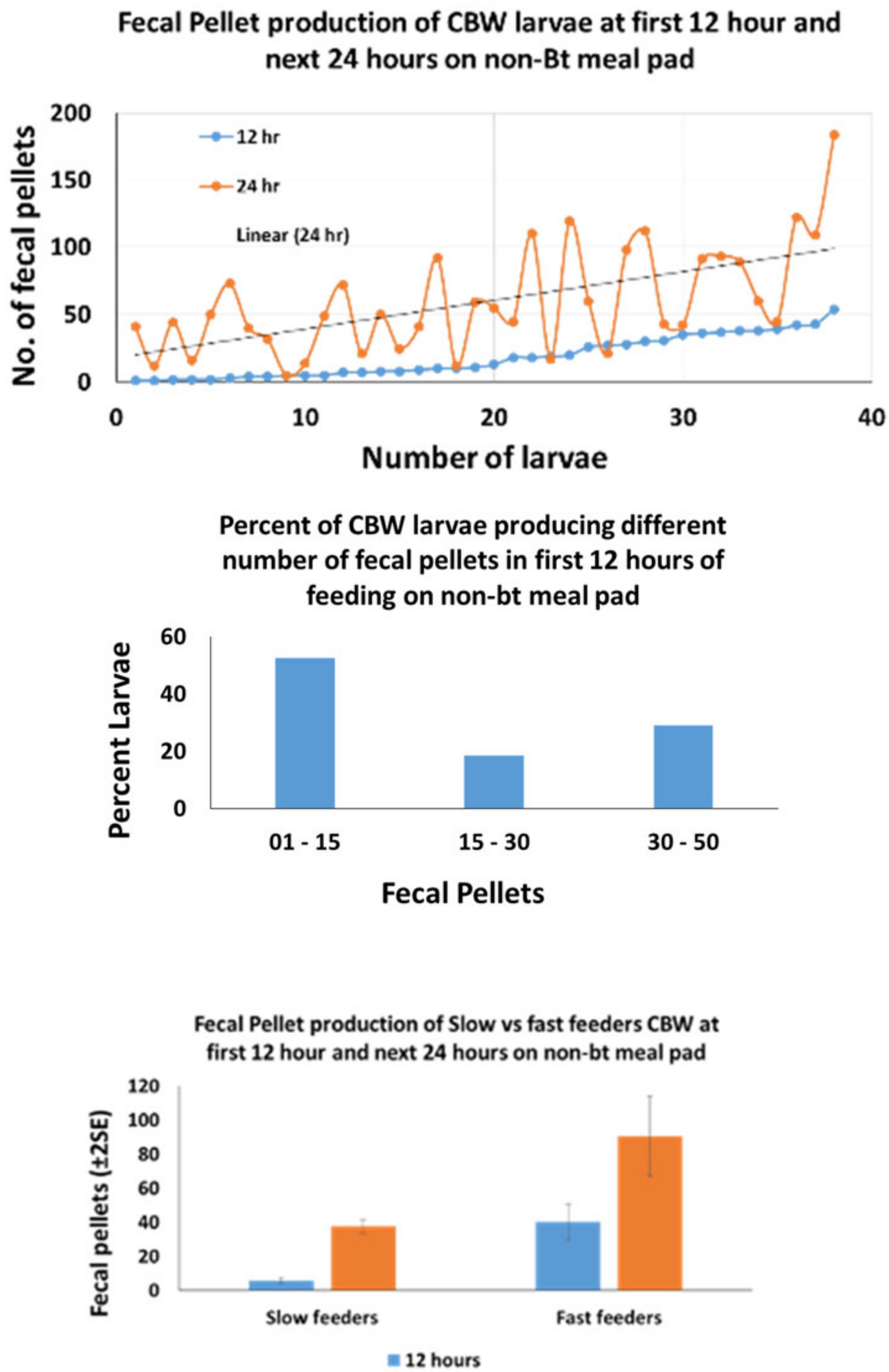


Figure 4. Fecal production rate per neonate at 25 ºC for neonates during their first 12 h as a larva versus their next 24 h for bollworms on artificial diet without Bt (top). Based on these predictive data, larvae can be sorted into a low and high feeding rate (middle and bottom).

Impact of Feeding Rate at a Constant Temperature on Susceptibility to MVPII

Fig. 5 shows the difference between fecal production on artificial diet (with no Bt) versus fecal production when a diagnostic dose of MVPII is added to the diet for slow versus fast feeding cotton bollworms over a 24 h incubation period at 25 ºC. The fold difference was 7.87-fold for slow feeders and was less for fast feeders (3.78-fold). This was consistent with our hypothesis that Bt susceptibility is reduced at higher feeding rates. Further replication is needed and similar studies needed for other caterpillar species to validate these findings.

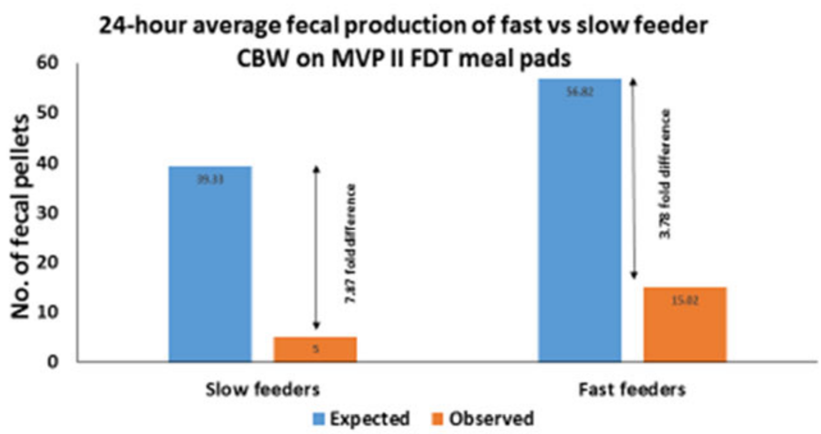


Figure 5. Fecal production for cotton bollworm slow and fast feeders over a 24 h incubation period on artificial diet without Bt (expected) versus with a diagnostic dose of MVPII in the diet.

Summary

We present evidence that increased feeding rates in tobacco budworm neonates could be a mechanism for reduced susceptibility to Bt toxins. Natural variations in feeding rates were reported for tobacco budworms and cotton bollworms, where higher feeding rates were correlated with reduced susceptibility to Bt in artificial diet. Reduced insect rearing temperature for a variety of temperature ranges also reduced insect feeding rates and increased Bt susceptibility for the cotton bollworm, and the same was found in a single temperature range for the tobacco budworm. A budworm strain selected for Bt resistance in the laboratory had a higher feeding rate than the parent strain and the same occurred for a field collected Bt resistant fall army worm strain compare to a Bt susceptible strain of the same species on artificial diet without Bt. Furthermore, when slow and fast feeding bollworms were exposed to Bt, the fast feeders appeared to be less susceptible to Bt.

Mechanism of decreased susceptibility.

One possible mechanism for a decreased susceptibility to Bt toxin 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 proteins (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 posterior 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 tolerance/resistance by insects in the field, the application of transgenic plant technologies could potentially be selecting for caterpillar populations with increased feeding rates which appears to be supported by our studies with Bt resistant fall armyworms.

Mechanism in perspective and impact of mechanism on caterpillars in cotton. The relative importance of feeding rates on Bt tolerance/resistance versus other potential mechanisms for tolerance/resistance is unknown. If nothing else, it provides an additional approach by which insects like the bollworm might survive current pyramided cotton technologies at a higher level and promote the evolution of potentially other resistance mechanisms of more significance. Worst case, feeding rates could be a method for broad high cross resistance to protein toxins and/or produce insects by selection with Bt crops that feed faster and potentially cause more plant damage than the susceptible strain on non-Bt plants. The possibility of increase tolerance of caterpillars to Bt at higher temperatures is also interesting for two reasons: (i) increased temperatures due to climate change could increase insect survival in the field; and (ii) differences in temperature within the cotton canopy might affect insect positioning on the plant.

It should be noted that if increased feeding rate results in susceptibility to Bt toxins in the field, insecticides with contact activity should remain effective to control these potentially fast feeding resistant populations. Our data stresses the need for growers/consultants to closely monitor their Bt crops and apply alternative controls when caterpillar pests are above threshold.

Acknowledgments

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