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Title: Nutrition as a neglected factor in insect herbivore susceptibility to *Bt* toxins

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Abstract

The widespread global adoption of *Bt* crops elevates concerns about the evolution of *Bt* resistance in insect pest species. Current insecticide resistance management (IRM) strategies focus solely on genetic variation as a causal factor in the evolution of resistance, but ignore the role that environmental factors, such as nutrition, may play. In this opinion paper, we discuss the benefits that insect herbivores gain from consuming foods with protein-carbohydrate content that matches their self-selected protein-carbohydrate intake, and show that even within monocultures there is ample opportunity for insect herbivores to regulate their macronutrient intake. Next we review new data that show that dietary protein and carbohydrates can: 1) have predictably strong effects on the survival and performance of caterpillars challenged with *Bt* toxins, and 2) mediate plasticity in susceptibility to Cry1Ac, which can account for large differences in LC₅₀ values. Nutrition-*Bt* interactions such as these have important implications for IRM, particularly given that diet-incorporated *Bt* bioassays commonly use artificial diets that vary substantially from their self-selected optimal diets, which likely results in underestimates of resistance in the field. Failing to bioassay larvae on ecologically-relevant diets can seriously confound the results of *Bt* resistance monitoring bioassays and undermine our ability to detect resistance in the field.

Graphical Abstract

Insect herbivores, including pest species targeted by *Bt* crops, actively regulate their protein-carbohydrate intake. These intake targets are functionally optimal.

There is significant variation in plant protein and carbohydrate content within and between plants, including agricultural crops. The data shown below are for *Bt* cotton.

Genetic factors are generally assumed to be responsible for the presence of resistant phenotypes. In this review we argue that environmental factors (plant nutrients) can mediate resistance through effects on gene expression. Insect herbivores feeding optimally are more resilient to *Bt* toxins.

Introduction

Since their introduction in 1996, genetically-modified *Bt* crops have revolutionized agriculture. *Bt* crop acreage has increased over 60-fold in the last 2 decades, currently exceeding 1 billion acres worldwide [1]. With this sharp increase in *Bt* crop usage, concerns about *Bt* resistance are growing. Presently, incidents of field-evolved resistance to *Bt* have been reported in 5 of the 13 pest species examined [1]. Current methods for slowing the evolution of resistance include: (1) producing *Bt* lines that express a high lethal dose of toxin and/or pyramided lines that express two different Cry toxins simultaneously, (2) controlling the spread of resistant genotypes by planting non-*Bt* refuges to encourage hybridization between resistant and susceptible individuals, and (3) early detection and quick mitigation of resistance by monitoring the dose-response of field-collected larva over time, particularly in regions where reductions in field efficacy are apparent.

The overriding assumption in *Bt* resistance management is that genetic factors are primarily responsible for the presence of resistant phenotypes [2,3,4]. This is true despite the fact that the genetic components of field resistance are rarely identified. An alternative to this gene-centric view is that environmental factors can also mediate resistance through effects on gene expression. This is a phenomenon we refer to as “environmentally-mediated resistance”. Interactions between genes and their environment can permit a single genotype (individual) to produce a range of phenotypes across different environmental conditions; a phenomenon referred to as phenotypic plasticity.

Nutrition is of fundamental importance to all animals, including insect herbivores [5,6], but has been neglected as a factor contributing to variation in insect herbivore susceptibility to *Bt* toxins. Plant nutrient content, particularly protein and carbohydrates, has been shown to be both

spatially and temporally variable [7,8,9], meaning that insect herbivores forage in a highly heterogeneous nutritional landscape. There is also strong evidence that the absolute amounts and ratios of protein and carbohydrates in insect herbivore diets strongly affect their performance, including growth rate and reproduction [5,10,11,12,13,14], as well as their tolerance to plant toxins [15,16] and immunological challenges [17,18,19].

So how might nutrition affect susceptibility to *Bt* transgenic crops? In Figure 1 we show a general phenotypic plasticity (genotype x environment interaction) model applied to variation in susceptibility to *Bt*. The usual expectation in resistance monitoring – that differences in larval performance are entirely a function of genotype – is shown in panel (a). In contrast, panel (b) shows that a genotype characterized as susceptible on one diet can exhibit reduced susceptibility mirroring that of a constitutively-resistant phenotype when it is reared on a high quality diet (e.g., the protein-carbohydrate is more balanced relative to that insect's protein-carbohydrate requirements). Knowledge of such nutritionally-mediated effects can help to explain variation in susceptibility, and aid in the development of more robust resistance monitoring assays in the lab that reflect insect performance in the field.

Despite the evidence that plant protein-carbohydrate profiles can be highly variable, and that food protein-carbohydrate profile impacts herbivore tolerance to plant toxins and immune responses, the effect of nutrition on *Bt* susceptibility is not well understood. This lack of attention is particularly noteworthy in agricultural systems where nutritionally-mediated variation in *Bt* susceptibility may have significant economic consequences. The aim of this review is to discuss how insect nutrition relates to *Bt* crops, and the implications for *Bt* resistance monitoring and management. We do this by highlighting recent work that examines the effects of food protein-carbohydrate content on Cry1Ac toxicity in caterpillars. We also draw on studies

that compare and contrast the feeding behavior and performance of *Bt*-resistant and *Bt*-susceptible strains. A critical implication of the nutritional effects that we highlight is that most widely used diets in *Bt* resistance monitoring assays for *Helicoverpa zea*, and potentially other herbivores, are nutritionally sub-optimal and ecologically unrealistic, likely obscuring the detection of both phenotypic and genetic variation for resistance when it is mediated by the nutritional environment.

Optimal nutrition in a nutritionally heterogeneous environment

To understand how nutrition may impact an insect's susceptibility to *Bt* toxins, we first need to know which nutrients are needed and the extent to which some are prioritized over others. All insect herbivores, including caterpillars and beetles that are common target pests on plants containing *Bt* toxins, require the same broad suite of nutrients [20]. These include amino acids (mostly obtained from dietary protein), carbohydrates (sugars and starch), lipids (fatty acids, phospholipids and sterols), vitamins and minerals. Lipids, vitamins and minerals are classified as micronutrients because they occur in plants at low levels, and generally insect herbivores need only small amounts. With the possible exception of sterols [21], micronutrients are not considered limiting for insect herbivores. In contrast, dietary protein and digestible carbohydrates are needed in larger amounts, and are considered limiting nutrients for insect herbivores because when they occur at low levels in plants they negatively affect fitness [22]. However, because insect herbivores (indeed all animals) require multiple nutrients simultaneously, optimal performance is only realized when nutrient levels are appropriately balanced [5,6]. Given the importance of protein and carbohydrates to insect herbivores, many species have been examined with respect to their ability to actively and simultaneously regulate

these two nutrients [5,23]. Two key findings have emerged. First, most insect herbivores tightly regulate their protein-carbohydrate intake, and this “intake target” is functionally optimal [12,13]. Second, the protein-carbohydrate ratio that leads to optimal fitness is often species specific [5,12,17,24].

Protein-carbohydrate intake targets have been identified for a number of caterpillars that are pests of *Bt* crops (Figure 2). These intake targets are important because they underlie the foraging decisions these caterpillars are making with respect to regulating, and prioritizing, their protein and carbohydrate intake. This figure also highlights three other important points. First, the protein-carbohydrate intake target reported by Waldbauer et al. [25] for *H. zea* (a P:C ratio of 4:1) is an extreme outlier. Deans et al. [26] recently revisited protein-carbohydrate regulation in *H. zea*, using the experimental approach of the geometric framework for nutrition [6], and found a self-selected P:C ratio of 1.6:1. As seen in Figure 2, the value reported by Deans et al. [26] is more inline with what has been reported for other caterpillars. Second, as reported by Shikano and Cory [27], protein-carbohydrate intake targets can differ between *Bt* susceptible and resistant lines. This suggests that selection for *Bt* resistance can also affect nutritional physiology. Third, a comparison of the four *Spodoptera* species indicates that closely-related species can have very different protein-carbohydrate intake targets. The sister species *Heliothis virescens* and *H. subflexa* also exhibit very different protein-carbohydrate intake targets [17], as do grasshoppers from the genus *Melanoplus* [12]. Thus, intake targets for one species should not be inferred based on relatedness.

Species-specific and strain-specific protein-carbohydrate intake targets suggest that protein-carbohydrate regulation is a product of selection. In the field, the ability to regulate protein-carbohydrate intake is particularly valuable if an insect experiences a high level of

protein-carbohydrate variability within its lifetime. Generalist insect herbivores that are highly mobile, and include in their diet (at the individual level) plants from multiple plant families, experience a very heterogeneous protein-carbohydrate landscape [9]. But what about insect herbivores that are specialists, or feed in monocultures? Studies that examine intra-plant variation in protein and carbohydrate levels are relatively rare, but it has been investigated in cotton, a key *Bt* transgenic host of *H. zea*. Within plant protein-carbohydrate content in cotton is highly variable at a range of spatial and temporal scales – between varieties, between tissues within a plant, over plant development, and across different growing environments [28] (C Deans, PhD thesis, Texas A&M University, 2015; Dean et al., unpublished(a)). For example, different tissues in a single cotton plant can exhibit a 4.5-fold difference in P:C ratio and a 3-fold difference in total macronutrient concentration within the same developmental stage (C Deans, PhD thesis, Texas A&M University, 2015; Deans et al., unpublished(a)). There were also strong differences in tissue macronutrients over time, with leaf P:C ratio and total macronutrient concentration declining as much as 60% throughout plant development. Showler and Moran [28] also documented an almost 200% increase in protein and a 250% increase in carbohydrate content between well-watered and water-stressed cotton leaves. These data suggest that even in agricultural monocultures with virtually no plant diversity, insect herbivores are foraging in a highly heterogeneous nutritional landscape.

Interactions between food protein-carbohydrate content and Cry1Ac

Several studies have connected food protein-carbohydrate content with the ability of insect herbivores to cope with different stressors, including plant toxins and immunological challenges. A highly relevant example for this review is Simpson and Raubenheimer [15], which showed

how food protein-carbohydrate content mediates the effects of tannic acid (a plant allelochemical) on locusts (*Locusta migratoria*). In this study, locusts suffered low mortality, even at high tannic acid concentrations, when they were reared on foods that had optimal protein-carbohydrate balance. However, mortality increased as food protein-carbohydrate content became more nutritionally unbalanced. Furthermore, on extremely unbalanced diets, high mortality was observed at low tannic acid concentrations. In a similar fashion to locusts, *H. zea* was more resilient to Cry1Ac when reared on a diet matching their self-selected P:C ratio, exhibiting significantly higher survivorship and associated LC₅₀ values when challenged with lethal doses of Cry1Ac (C Deans, PhD thesis, Texas A&M University, 2015; Deans et al., unpublished(b)). Feeding on an optimal P:C ratio also improved larval performance at sub-lethal Cry1Ac concentrations. Figure 3 shows that there were no differences in time to pupation in the absence of Cry1Ac, but at a sub-lethal dose of 0.6 ppm Cry1Ac, the diets that most closely matched *H. zea*'s intake target yielded the fastest developmental time, particularly for the more concentrated diet.

More research is needed to clearly resolve the relative importance of diet P:C ratio and total macronutrient concentration, but it is clear that these two dietary components mediate the effects of Cry1Ac in *H. zea* with P:C ratio playing the primary role (C Deans, PhD thesis, Texas A&M University, 2015; Deans et al., unpublished(b)). Opert et al. [29] also used *H. zea* to examine interactions between insect nutrition and Cry1Ac. They also showed that food protein-carbohydrate content can affect *H. zea* susceptibility to Cry1Ac in a genetically-susceptible strain, but it is difficult to interpret the ecological significance their results because their optimal reference diet was based on the 4:1 P:C ratio from Waldbauer et al. [25], which has since been shown to be inaccurately high [26]. Perhaps as a consequence of this, two of their four protein-

carbohydrate treatments reflect high P:C ratios (4:1 and 9:1) that are rarely experienced by *H. zea* on cotton in the field (C Deans, PhD thesis, Texas A&M University, 2015; Deans et al., unpublished(a)). Finally, Shikano and Cory [30] examined how food protein-carbohydrate content affected the Cry1Ac LC₅₀ in the caterpillar *Trichoplusia ni*. They found that the LC₅₀ of resistant strains, but not susceptible strains, decreased when fed diets with excess protein. Opert et al [29]) also showed differential responses between resistant and susceptible *H. zea* strains, but in this case nutritional variation failed to have an effect on the resistant strain as it did with the susceptible insects.

Though specific plant tissues can be characterized in terms of P:C ratios, an individual insect foraging in the field is not restricted to a specific tissue type, or ratio, and can choose from available options at a finer scale to regulate protein and carbohydrate intake with respect to its nutritional intake target, which may or may not change in the presence of toxins. Evidence in locusts suggests that protein-carbohydrate foraging decisions made in the presence of toxic compounds are strongly influenced by the nutritional quality of their food. Behmer et al. [16] showed that when locusts have carbohydrate-biased food paired with protein-biased food they tightly regulate their protein-carbohydrate intake. If the protein-biased food, but not carbohydrate-biased food contains tannic acid, protein-carbohydrate intake is still tightly regulated. In contrast, when the carbohydrate-biased food, but not the protein-biased food contains tannic acid, they eat little of the diet with tannic acid and select a protein-biased intake. For lepidopteran species, Cry toxins generally have a deterrent effect on larval feeding [31,32]. Despite this, no studies have documented how Cry toxins explicitly modify protein-carbohydrate regulation, relative to treatments that lacked Cry toxins. Interestingly, Gore et al. [33] reported significant differences in *H. zea* larval behavior on *Bt* versus non-*Bt* plants, with a higher

proportion of larva on *Bt* plants infesting flowers and bolls. Flowers and bolls generally have lower expression levels of Cry1Ac than leaves and squares [34,35,36]; however, these tissues may also have different nutritional profiles. While limited macronutrient data is available for cotton flowers, Hedin and McCarty [37] have shown that cotton anthers are a high-P resource, and developing seeds in cotton bolls have both a high total macronutrient concentration and a P:C ratio of 1.6:1 that perfectly matches the intake target for *H. zea* [26](C Deans, PhD thesis, Texas A&M University, 2015; Dean et al., unpublished(a)).

Nutritional implications for *Bt* resistance and monitoring

Current definitions of resistance fail to acknowledge the effect that environmental factors, such as nutrition, can have on the efficacy of *Bt* crops [2,3,4]. Not only does nutritional plasticity have the potential to impact the evolution of resistance via survival and sub-lethal effects on pest species that violate the assumptions of high dose and low initial resistance allele frequency (*H. zea* [38]; *H. armigera* [39]; *Heliothis virescens* [40,41]; *Pectinophora gossypiella* [42,43]), but nutritionally-mediated effects on susceptibility may also explain a large portion of the variability in *Bt* efficacy observed in the field and across lab studies. Much more work is needed to fully understand the relationship between nutrition and the various forms of resistance, but the implications of the available data for insecticide resistance management (IRM) are perhaps the most critical in the near term.

Detecting resistance in field populations is at the forefront of IRM. Although plant-based bioassays are sometimes used, diet-incorporation bioassays are much more common. For these assays, neonate mortality is assessed across a range of Cry concentrations incorporated into an artificial diet. Typically, commercially-available rearing diets are used in these assays, often

because they are inexpensive and easy to make. However, new evidence suggests that, although these diets are adequate for rearing laboratory cultures, they are likely not nutritionally relevant to the field.

Using choice tests, [26] showed that *H. zea* selected for a slightly protein-biased P:C ratio of 1.6:1. When larval performance was measured across a range of diet P:C ratios with Cry1Ac incorporated, those reared on the diet closest to this intake target showed the highest survival, best overall performance, and highest LC₅₀ concentration (C Deans, PhD thesis, Texas A&M University, 2015; Deans et al., unpublished(b)). However, the P:C ratios of artificial diets used in the most recent *Bt* bioassays on *H. zea*, as well as the two most commonly used commercial rearing diets for *H. zea* (Southland Products and Frontier Agricultural Sciences (formerly Bio-Serv)), are all extremely carbohydrate-biased (Table 1). Not only are these P:C ratios substantially lower than the optimal 1.6 ratio, but in Deans (PhD thesis, Texas A&M University, 2015; Deans et al., unpublished (b)) the carbohydrate-biased diets tested in this range showed the lowest survival, performance, and LC₅₀ for insects challenged by Cry1Ac. However, survival data from Orpet et al. [29] suggests that diet-Cry interactions may vary between insect populations.

Protein-carbohydrate intake targets for insect herbivores are based on feeding behavior, so they provide the best representation of foraging behavior in the field. A discrepancy in food protein-carbohydrate content between the diet larvae consume in the field and the diet used in resistance bioassays has the potential to seriously confound the results of resistance monitoring efforts. For example, *H. zea* larvae that appear to be resistant in the field could actually test as susceptible in lab bioassays simply because they have been tested on a sub-optimal diet. The available nutritional data suggest that current resistance monitoring assays, based on sub-optimal

carbohydrate-biased diets, are likely overestimating susceptibility in field populations of *H. zea* by greater than two orders of magnitude (Dean et al., unpublished(b)). Insect mortality in these assays is a consequence of being stressed by both a suboptimal diet and *Bt* toxins. It does not accurately reflect their susceptibility while foraging to achieve their nutritional intake target under field conditions and can lead to the erroneous conclusion of low levels of resistance. It's also likely that similar nutritional confounds occur in diet-based assays for other pest species. Ultimately, the inability to accurately detect reduced susceptibility in agricultural pest populations compromises the effectiveness of IRM programs.

Conclusions

While the potential economic impact of nutritionally-mediated plasticity in *Bt* susceptibility might appear minor in comparison to that of genetically-determined resistance, such plasticity may not only help to explain observed variability in the efficacy of *Bt* crops, it can also be an adaptive intermediate stage in the rapid evolution of genetically-determined resistance [43]. We have discussed the strong effect that nutrition can have on *Bt* susceptibility as an example of environmentally-mediated resistance and highlighted some negative consequences of not accounting for nutrition in IRM monitoring. Despite the overall success of *Bt* technology, field failures can occur without warning. Using ecologically-relevant diets informed by insect physiology for resistance monitoring that do not overestimate susceptibility to *Bt* toxins will improve the ability to detect resistance in a timely manner as it unfolds in the field, enabling proactive responses to protect the efficacy of the technology before it is too late.

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This paper looks at the effects of diet P:C ratio on *Bt* susceptibility in a *Bt*-resistant and *Bt*-susceptible strain of cotton bollworm (*Helicoverpa zea*). Results show that diet P:C only had an effect on performance when the *Bt*-susceptible strain was challenged with Cry1Ac, while diet had no effect on the performance of the *Bt*-resistant strain. It should be noted, however, that the diets tested in this study are centered around a 4:1 P:C diet being optimal (Waldbauer et al., 1984), which has since been revised to a P:C or 1.6:1 by Deans et al. (2015).

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The authors show that Cry1Ac acts as a feeding deterrent in both a *Bt*-resistant and *Bt*-susceptible strain of cotton bollworm (*Helicoverpa zea*). It should be noted, however, that the diets tested in this study are centered around a 4:1 P:C diet being optimal (Waldbauer et al., 1984), which has since been revised to a P:C or 1.6:1 by Deans et al. (2015).

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Table 1. The P:C ratios and total macronutrient concentrations for the rearing diets cited in the most recent studies on *Bt* resistance in *H. zea*, as well as commonly used commercially-available rearing diets for *H. zea*. If available, the original citation for the diet recipe is given in parentheses under Diet Type, along with the main ingredients.

Citation	Diet Type	P:C Ratio	Total Macronutrients (%)
Luttrell et al. (1999)	corn, soy flour, wheat germ (King et al., 1985)	1:2	62.5
Ali et al. (2006)	corn, soy flour (Burton, 1970)	1:3	70.9
Ali et al. (2007)	pinto bean, yeast wheat germ (Burton, 1969)	1:2	74.5
Southland Products	soy flour, wheat germ	1:2.3	62.8
Frontier Agricultural Sciences	soy flour, wheat germ	1:2.5	63.2

Figure Legends

Figure 1. A general phenotypic plasticity model showing reduced-susceptibility to *Bt* mediated by the nutritional environment. Panel (a) shows a genetically-determined effect, while panel (b) shows an environmentally-determined effect.

Figure 2. The intake targets for various caterpillar species targeted by *Bt* crops, including: *Spodoptera exempta* [10], *S. exigua* [46], *S. littoralis* [10], *S. litura* [47], *Plutella xylostera* [45], *Trichoplusia ni* (a resistant (R) and susceptible (S) strain)[30], *Heliothis virescens* [13,17], *Helicoverpa zea* [26] and Waldbauer et al. [25] listed separately.

Figure 3. Data taken from Deans (2015) showing % pupating and time to pupation for larvae reared on diets that differ in P:C ratio and total macronutrient concentration in the absence of Cry1Ac (a) and with 0.1 ppm (b) and 0.6 ppm (c) of Cry1Ac incorporated into the diets. Diet effects were only significant for the 0.6 ppm treatment ($X^2=13.49$, $df=3$, $P<0.004$). Different letters denote significant post-hoc differences at the $\alpha=0.05$ level.

Figure 1

Figure 2

Figure 3