THE EFFECTS OF BT CORN ON RUSTY CRAYFISH (*ORCONECTES RUSTICUS*) GROWTH AND
SURVIVAL

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A Thesis

Submitted to the Graduate College of Bowling Green
State University in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

August 2014

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ABSTRACT

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Bt crops are one of the most commonly utilized genetically modified crops worldwide. Bt crops contain a gene that is derived from the bacteria *Bacillus thuringiensis*, which produces the Cry1Ab toxin. Bt corn that contains the Cry1Ab toxin is used throughout the Midwest United States to control crop pests such as the European corn borer (*Ostrinia nubilalis*). Headwater streams in regions known for intensive agriculture receive Bt corn detritus following the fall harvest, which is then consumed by a diverse community of stream invertebrates. The rusty crayfish (*Orconectes rusticus*) is an common invertebrate detritivore in these headwater streams. Both isogenic and Bt corn were grown under the controlled environmental conditions of a greenhouse and, following senescence, were tested for nutritional equality. Rusty crayfish were exposed to one of several detrital treatments composed of Bt corn, Bt corn plus American sycamore (*Platanus occidentalis*), isogenic corn, isogenic corn plus *P. occidentalis*, or *P. occidentalis* for eight weeks. Crayfish were housed in live streams with a water temperature of 12.8°C and a 12:12 photoperiod. Survival and growth of animals within each experimental treatment were monitored each week. After eight weeks of exposure, there was a statistically significant difference in growth and survival between crayfish in Bt and isogenic treatments. These results suggest that the Bt and isogenic corn were of equivalent nutritional value, but Bt corn does have a toxic affect on rusty crayfish during long-term exposure.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Paul Moore and members of the Laboratory for Sensory Ecology at Bowling Green State University for suggestions, comments, and assistance in data collection. I would like to thank Frank Schemenauer for caring for the corn in the greenhouse. I would also like to thank Dr. Jeffrey Miner at Bowling Green State University for the use of his laboratory equipment.

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INTRODUCTION

Intensive agriculture adjacent to headwater streams of the Midwestern United States has resulted in increased run-off, changes in hydrology, and alterations in the composition of allochthonous material in these streams. Row crop agriculture removes native riparian vegetation, which is an important link between aquatic and terrestrial ecosystems (Gregory et al. 1991). Riparian vegetation buffers streams from organic pollutants and reduces the kinetic energy of moving water (Naiman & Decamps 1997; Tabacchi et al. 2000). The removal of riparian vegetation alters the amount and composition of organic detritus entering streams, which have different chemical constituents (Tabacchi et al. 2000). As a result of increased row crop agriculture, riparian zones contain corn, wheat, soybean and other row crop agriculture plants.

Tissues from monoculture crops such as leaves, cobs, husks, and stalks of corn are commonly found in the stream channel of Midwestern streams (Douville et al 2007; Griffiths et al. 2009; Jensen et al. 2010; Rosi-Marshall et al. 2007; Tank et al. 2010). Corn tissue inputs can be found in these headwater streams following the harvest in late fall and peak in February or March (Douville et al. 2007; Rosi-Marshall et al. 2007). A study conducted in Indiana found corn stalks, cobs, or leaves in 86% of sampled streams (Tank et al. 2010). Given the increased use of genetically modified crops in the last decade, a larger percentage of the detrital inputs into aquatic habitats will be from genetically modified cultivars.

Genetically modified organisms are organisms that possess a gene that has been transferred from another organism in order to produce a desirable trait. These traits can include resistance to pests, disease, herbicides, and environmental stressors (Nap et al. 2003). Genetically modified agricultural crops with these traits have become prevalent throughout the world. Many of the available genetically modified crops are important to U. S. agriculture such

as cotton, corn, potato, soybean, tobacco, sugarbeet, squash, rice, linseed and tomato (Altieri 2000; Castagnola & Jurat-Fuentes 2012; Garcia & Altieri 2005; Nap et al. 2003). With the increasing popularity of genetically modified crops and changes in surface water hydrology, allochthonous input into streams will contain an increasing amount of compounds from genetically modified crops.

Bt corn is one of the most common genetically modified crops with approximately 52% of all the corn planted in the United States containing the Bt trait (NASS 2012). Bt corn contains the Cry1Ab toxin, which increases the plant's defenses against insect herbivory. The Cry1Ab endotoxin is derived from the soil bacteria *Bacillus thuringiensis* (Aronson & Shai 2001; Bravo et al. 2007). When consumed the Cry1Ab endotoxin binds to membrane receptors in the midgut of target pests, which then causes cells to lyse, increasing the hemolymph potassium concentration (Bravo et al. 2007; Whalon & Wingerd 2003). The increased potassium concentration results in insect death from paralysis, starvation, and septicemia (Whalon & Wingerd 2003). The Cry1Ab endotoxin is believed to be more selective than chemical pesticides at killing insect pests such as the European corn borer (*Ostrinia nubilalis*), corn earworm (*Helicoverpa zea*), and the southwestern corn borer (*Diatraea grandiosella*) (Mendelson et al. 2003). However, recently there has been discussion about the selectivity of the Cry1Ab toxin (Hilbeck & Schmidt 2006).

While the Cry1Ab toxin was originally thought to be highly selective, some researchers now argue that the mode of action in non-target organisms is not fully understood (Hilbeck & Schmidt 2006). There are a number of factors that are also believed to influence the selectivity of the Cry1Ab toxin. Once the Cry1Ab toxin is activated, plant enzymes potentially act as cofactors and alter the toxin (Li et al. 2007). Infectious diseases (Dubois & Dean 1995), as well as biotic

and/or abiotic stressors, (Koppenhöfer & Kaya 1997; Kramarz et al. 2007) have been shown to alter toxicity. In some organisms the presence of gut bacteria appears to influence toxicity (Broderick et al. 2006). In addition to questionable selectivity, the Cry1Ab toxin has been documented to impact non-target organisms across multiple taxa.

Research has established potential impacts on many terrestrial organisms including monarch butterfly (*Danaus plexippus*) populations (Losey et al. 1999; Sears et al. 2001; Kaplan 2002; and Dively et al. 2004), soil bacteria (Baumgarte & Tebbe 2005), slugs (Hönemann & Nentwig 2010), ladybirds (Schmidt et al. 2009), common green lacewings (Hilbeck 2001), earthworms (Zwahlen et al. 2003), and land snails (Kramarz et al. 2009). While knowledge on Bt toxin impacts on terrestrial ecosystems has been assessed (Barton & Dracup 2000; Conner 2003; Rose & Dively 2007), there is uncertainty regarding the impacts of Bt corn detritus on stream ecosystems. Bt corn detritus regularly enters streams, yet little is known about interactions between Bt toxin and aquatic consumers (Castagnola & Jurat-Fuentes 2012).

The interactions between Bt corn detritus and aquatic ecosystems have been investigated using the model organism *Daphnia magna* and a select group of macroinvertebrate insects. Experiments with *Daphnia magna* have indicated that Bt corn can reduce growth and survival (Bøhn et al. 2008; Bøhn et al. 2010). Additional studies have found that trichopteran species experience reduced growth rates and reduced survivorship when fed Bt corn detritus (Chambers et al. 2010; Rosi-Marshall et al. 2007). Jensen et al. (2010) documented similar responses to Bt detritus in crane flies and isopods, but attributed these responses to tissue differences in corn tissues rather than the Cry1Ab toxin. Macroinvertebrate insects such as caddisflies, crane flies, and isopods are common organisms in headwater streams, but are accompanied by a diverse group of additional detritivores that may also consume the Cry1Ab toxin.

Detritivores are an important link in stream food chains by processing allochthonous material from nearby agricultural fields that are transported into the stream channel. Shredders perform the essential task of breaking down large-scale agricultural tissue into fine particulate organic matter (FPOM) that can be consumed by additional aquatic species (Cummins et al. 1989). Macroinvertebrate shredders generally include insects such as caddisflies, stoneflies, dragonflies, and mayflies (Cummins et al. 1989), but also include non-insect invertebrate detritivores such as isopods, amphipods, snails, and crayfish that breakdown coarse particulate organic matter (CPOM) (Anderson and Sedell 1979). In eutrophic headwater streams crayfish are more abundant than many macroinvertebrates species and play a significant ecological role in processing agricultural detritus (Hynes 1970).

Crayfish are considered keystone species by having polytrophic interactions within a wide variety of aquatic ecosystems throughout North America. In some habitats, crayfish have been documented to comprise 50% of the macroinvertebrate biomass (Momot 1995) and are major consumers of plant material (Momot et al. 1978). In addition to shredding and consuming detritus, crayfish are important predators of macroinvertebrates such as rotifers, cladocerans, ostracods, chironomids, and other arthropods (Momot et al. 1978). Finally, crayfish are key prey items for important commercial and recreational freshwater fish such as centrarchid species (Olson & Young 2003). Given these attributes of crayfish ecology, crayfish function as keystone species by processing allochthonous carbon, functioning as predators and as prey species (Momot et al. 1978). Among this diverse group of ecologically significant crayfish is the genus *Orconectes*.

The genus *Orconectes* accounts for approximately 25% of the crayfish species in North America (Holdich 2002). The rusty crayfish (*Orconectes rusticus*) is a member of this genus that

is native to the Ohio River Basin (Olden et al. 2006; Taylor et al. 1996). *O. rusticus* can be found in lakes, rivers, ponds, and streams within its ranges (Taylor et al. 1996). Populations of *O. rusticus* are widely distributed within the Midwestern United States. Consequently, there exists a high possibility that *O. rusticus* populations are exposed to the Cry1Ab protein in the form of detrital runoff following fall harvests of Bt corn. Given that crayfish are significant detritivores in headwater streams throughout the corn-belt, any potential negative impact on their populations could have large-scale impacts on stream ecosystems. The objective of this study was to quantify the growth and survival of *O. rusticus* exposed to Bt corn detritus.

METHODS

Crayfish collection and storage

Male and female adult *O. rusticus* with a mean ($\pm$ SEM) carapace length of 3.0 ± 0.14 cm were collected from the Portage River in Wood County, OH between the fall of 2012 and the summer of 2013. Crayfish were stored in a temperature-controlled chamber with a 12:12 photoperiod and a recirculating flow through water source. Each individual was visually and mechanically isolated, while being fed rabbit pellets three times a week until trials began. Crayfish were starved one week prior to trials, to ensure a motivation to feed during trials.

Bt corn detritus

Bt corn (Cultivar ID# NG6143) and isogenic corn (Cultivar ID# NG6143) were obtained from Rupp Seeds (Wauseon, Ohio). Seeds were placed in Metro-Mix 852 topsoil and grown in the Bowling Green State University greenhouse throughout the winter and early spring under ambient light conditions (16 hours daily). Air temperature in the greenhouse ranged from 18.3 °C to 32.2 °C. Identical amounts of 10-10-10 fertilizer were applied weekly. Corn was allowed to grow until maturity (90-111 days). Once the corn matured and dried (129 days), a combination of leaves, stalks, and cobs were removed from the plants. These tissues were placed in a drying oven at 80 °C for 12-24 hours. Natural detritus was simulated using American sycamore (*Platanus occidentalis*) leaves, which are commonly found near headwater streams. The sycamore leaves were also dried at 80 °C for 12-24 hours. All dried detritus was preconditioned for one week in numbered glass vials filled with stream water collected from the Portage River (Wood County, OH).

Detrital nutritional quality and digestibility

Five Bt plants and five isogenic plants were removed at the base following maturity using pruners. From these ten plants, 60-70 grams of both leaf and stalk tissue were removed and placed in Ziploc bags. These particular tissue samples were then tested for carbon (C), nitrogen (N), and lignin content by the Dairy One Forage Testing Laboratory (Ithaca, New York). Bt and isogenic stalks were used in trials because they had the closest match based on both C:N and lignin content in order to standardize the nutritional value and digestibility of the food source (Table 1). Bt corn samples were taken following drying in order to test for the presence of the Cry1Ab toxin using Bt-Cry1Ab/1Ac ImmunoStrips® (Agdia Inc., Elkhart, IN) prior to trials.

Exposure systems

In order to test Bt corn impacts on growth and survival, crayfish were housed in 530 liter (213 x 61 x 56 cm) recirculating live streams (Frigid Units Inc. Model: LS-400) to simulate a lotic environment. All growth trials were conducted at a constant water temperature of 21°C. Crayfish were housed in separate BPA free Tupperware containers (9.2 x 9.2 x 7.5 cm). Given the recirculating nature of the streams, each stream contained a single detritus treatment.

Growth treatments

Crayfish used in all trials were size matched to within 10% of total body mass. All treatments lasted eight weeks and each crayfish was given 0.2 grams of detritus weekly in all treatments. Stalks were cut into smaller pieces using scissors until they were the correct weight. Detritus was weighed using a Sartorius scale. Each week crayfish were removed from the isolation containers to be weighed. Before being returned to isolation containers for another week, detritus was replaced with a new 0.2 grams of detritus of the appropriate variety. The

detritus from the previous week was placed in a drying oven for 12-24 hours at 80°C and weighed in order to determine total consumption.

Treatments

Detritus treatments were as follows:

- 1) Bt corn detritus (N=38)
- 2) Bt corn with *P. occidentalis* detritus (N=35)
- 3) Isogenic corn detritus (N=36)
- 4) Isogenic corn with *P. occidentalis* detritus (N=36)
- 5) *P. occidentalis* detritus (N=40)

In the Bt corn treatment all crayfish were fed 0.2 grams of Bt detritus. Crayfish in the Bt corn with *P. occidentalis* detritus treatment were fed 0.1 grams of Bt corn and 0.1 grams of *P. occidentalis* detritus. Crayfish in the isogenic corn treatment were fed 0.2 grams of isogenic corn detritus. Crayfish in the isogenic corn with *P. occidentalis* detritus treatment were fed 0.1 grams of isogenic corn and 0.1 grams of *P. occidentalis* detritus. Crayfish in the *P. occidentalis* treatment were fed 0.2 grams *P. occidentalis* detritus. All treatment groups were fed on a weekly basis.

Data analysis

Survival and growth were analyzed using a Kaplan-Meier log-rank test in the SPSS survival package. Growth was measured by monitoring the number of crayfish that molted each week. Detrital consumption had a non-normal distribution and was compared between treatment groups using multiple Mann-Whitney U tests with adjusted alpha levels.

RESULTS

Survival

The log rank test (Mantel-Cox) revealed a significant difference in survival between the Bt and isogenic treatments ($p < 0.01$). Significant differences were also found between the Bt and Bt mix treatments ($p < 0.01$), Bt and *P. occidentalis* treatments ($p < 0.01$), Bt mix and isogenic mix treatments ($p < 0.05$), isogenic mix and *P. occidentalis* treatments ($p < 0.05$), and the isogenic and isogenic mix treatments ($p < 0.01$). There was no significant difference between the Bt and isogenic mix treatments ($p > 0.05$), Bt mix and isogenic treatments ($p > 0.05$), isogenic and *P. occidentalis* treatments ($p > 0.05$), or the Bt mix and *P. occidentalis* treatments ($p > 0.05$). *O. rusticus* experienced 61% survival after eight weeks of exposure for the Bt treatment, 92% survival for the isogenic treatment, 91% survival for the Bt mix treatment, 67% survival for the isogenic mix treatment and 90% survival for the *P. occidentalis* treatment (Fig. 1).

Consumption

There was a significantly greater amount of detritus consumed in the Bt treatment compared to the Bt mix treatment ($p < 0.001$), isogenic mix treatment ($p < 0.001$), and *P. occidentalis* treatment ($p < 0.001$). Consumption of detritus in the isogenic treatment was significantly greater than the consumption in the Bt mix treatment ($p < 0.001$), isogenic mix treatment ($p < 0.001$), and *P. occidentalis* treatment ($p < 0.001$). Consumption in the Bt mix treatment was also significantly greater than the *P. occidentalis* treatment ($p < 0.001$). There was no significant difference in consumption rate of detritus between the Bt and isogenic treatments ($p > 0.005$), Bt mix and the isogenic mix treatments ($p > 0.005$), or isogenic mix and *P. occidentalis* treatments ($P > 0.005$). By the end of eight weeks *O. rusticus* consumed a weekly

average of 0.07 ± 0.002 g of detritus in the Bt treatment, 0.08 ± 0.002 g in the isogenic treatment, 0.06 ± 0.002 g in the Bt mix treatment, 0.05 ± 0.001 g in the isogenic mix treatment, and 0.05 ± 0.002 g in the *P. occidentalis* treatment (Fig. 2).

Growth

The log rank test (Mantel-Cox) revealed a significant difference in the number of *O. rusticus* that molted between the Bt and isogenic treatments ($p < 0.01$), Bt and isogenic mix treatments ($p < 0.01$), Bt mix and isogenic mix treatments ($p < 0.01$), isogenic and *P. occidentalis* treatments ($p < 0.01$), isogenic mix and *P. occidentalis* treatments ($p < 0.01$) as well as Bt mix and isogenic treatments ($p < 0.01$). Based on the percentage of *O. rusticus* that molted, the Bt treatment had the least growth with 5% of the animals molting within eight weeks (Fig. 3). The percentage of *O. rusticus* that molted after eight weeks of exposure was 5% for the Bt treatment, 39% for the isogenic treatment, 8% for the Bt mix treatment, 16% for the isogenic mix treatment and 10% for the *P. occidentalis* treatment (Fig. 3).

DISCUSSION

O. rusticus survival declined significantly when fed exclusively Bt corn stalks compared to isogenic corn stalks (Fig. 1). The growth of *O. rusticus* also decreased when fed exclusively Bt corn as compared to isogenic corn. Animals that were fed exclusively Bt corn stalks molted significantly less than their conspecifics that were fed isogenic corn stalks (Fig. 3). The influence of corn detritus on the growth and survivorship of the *O. rusticus* appears to have interactive effects when combined with other detrital inputs. When Bt corn was mixed with *P. occidentalis* leaves the survival of *O. rusticus* was significantly greater than the Bt corn treatment (Fig. 1). However, the survival of *O. rusticus* declined significantly when fed a mixture of *P. occidentalis* leaves and isogenic corn (Fig. 1). These results suggest that Bt corn is not equivalent to isogenic corn as a food source for *O. rusticus*.

Stream ecosystems can provide detritivores with a diverse group of allochthonous inputs, each of which has a unique chemical composition. While these chemical constituents alone may not have a direct detrimental effect when ingested by the consumer, interactions among various chemicals could have critical physiological implications by altering the conditions of the digestive tract (van Frankenhuyzen et al. 1985; Friberg et al. 1980; Havas 1980). This explanation is supported by the low survival of crayfish that fed on a mixture of isogenic corn and *P. occidentalis* detritus, while crayfish that fed each of these forms of detritus independently had high survival (Fig. 1). The change in survivorship between these two treatments could be attributed to differences in the nutritional quality of the detrital source, defense compounds, or even structural materials such as lignin and cellulose (Mattson 1980; Moran & Hodson 1989; Ostrofsky 1993; Swan & Palmer 2006). If the previous explanation is correct, then we would expect different mixtures of detritus present in streams to have interactive effects on crayfish

survival that do not exist when any of the detrital components are consumed independently. In addition to having direct physiological implications, the possibility exists that interactions between detrital sources could also alter the symbiotic gut bacteria community of *O. rusticus*.

Many detritivores, including *O. rusticus* are host to symbiotic gut bacteria. These symbiotic gut bacteria are essential to the digestion and absorption of nutrients found in allochthonous terrestrial inputs. Without these symbiotic bacteria *O. rusticus* would be unable to break down structural materials such as cellulose, reducing the amount of available nutrition (Gherardi et al. 1999). Currently, little is known about the digestive tract bacteria of *O. rusticus* and what conditions could alter species composition. However, the possibility exists that different detrital compositions could alter the environmental conditions that support vital symbiotic gut bacteria (Dittmer et al. 2012; Knapp et al. 2009; Zimmer et al. 2004). Changes to the quantity or species composition of symbiotic gut bacteria in *O. rusticus* could explain the reduced survival in the isogenic corn mixed with *P. occidentalis* treatment.

If stream ecosystems with heterogeneous mixtures of detritus alter the conditions of the *O. rusticus* gut, individuals may make feeding decisions based on the composition of detritus (Adams et al. 2003; Adams et al. 2005; Kominoski et al. 2007). Feeding behaviors that discriminate between different detrital inputs may give *O. rusticus* the ability to find food containing the desired nutrients or composition and, consequently, regulate gut conditions. Regulation of gut conditions would be advantageous when there are multiple detrital sources present, but would likely be limited in utility when stream detritus is homogenous in composition. Homogenous detrital composition in streams could be caused by low diversity terrestrial vegetation (e.g. agricultural monocultures) or the timing of fall inputs.

Our data demonstrate that, when no other detritus is present, Bt corn is not an equivalent food source when compared to isogenic corn for *O. rusticus* (Fig. 1). While the mechanism is currently unknown, our data also suggest Bt corn has the potential to increase the mortality of *O. rusticus* under certain environmental conditions. The primary environmental condition that could alter the effect Bt corn has on *O. rusticus* is the composition of detritus found in conjunction with Bt corn in streams. Variations in detrital composition could aid in determining the risk Bt corn poses to population of *O. rusticus*. In headwater streams that are adjacent to agricultural corn fields, the composition of terrestrial inputs and the corresponding risk to crayfish will not be fixed but rather vary both spatially and temporally (Abelho & Graca 1998; Benfield 1997; Hagen et al. 2010).

In addition to crayfish, other detritivores in headwater streams are exposed to Bt corn and other mixtures of allochthonous inputs. Invertebrate detritivores are generally grouped into functional feeding groups that include shredders, collectors, scrapers, piercers, and predators (Cummins & Klug 1979). Shredders are the first invertebrate group to encounter terrestrial vegetation in streams. Insect shredders such as amphipods, isopods, stoneflies, and some caddisflies perform a similar ecological role to *O. rusticus* in streams by converting CPOM into FPOM (Anderson & Sedell 1979; Cummins & Klug 1979; Cummins et al. 1989; Wallace & Webster 1996). Because invertebrate insect shredders perform a similar ecological role as *O. rusticus*, they may respond to the consumption of Bt corn and detrital mixtures in a similar fashion. If insect shredders do respond in a similar fashion, we would expect to see reductions in the survival and growth of insect shredders when exposed to Bt corn and interactive effects when they are exposed to multiple allochthonous inputs simultaneously. These effects would have the potential to alter the structure and composition of insect detritivore communities.

Allochthonous inputs in streams play an important role in the ecology of the ecosystem by providing a food base. In some streams, adjacent terrestrial vegetation provides the majority of organic matter to stream systems (Fisher & Likens 1973). Not only does riparian vegetation control the quantity of allochthonous material present in streams, riparian vegetation also controls the input type (Gregory et al. 1991). The type of allochthonous input is important because the chemical composition of each input can vary. This variation in chemical composition of allochthonous inputs has the potential to reduce consumer growth when detritus is low in nutritional value or high in compounds such as lignin, that makes the material difficult to digest (Swan & Palmer 2006). Consequently, reductions in consumer growth due to detrital inputs could potentially have bottom-up effects on stream ecosystems.

Stream invertebrates are the primary energy link between detritus and higher trophic levels. A reduction in the growth of stream invertebrates would reduce the total biomass at higher trophic levels (McIntosh et al. 2005; Nyström et al. 2003; Wallace et al. 1997). Primary consumers including populations of predatory stream invertebrates and fishes would experience a reduction in biomass. In addition to primary consumers, secondary consumers such as piscivorous fish communities would experience reductions in biomass. In conclusion, the introduction of Bt corn has the potential to alter stream food webs when introduced to streams independent of other detrital inputs. However, the introduction of multiple detrital inputs that enter streams simultaneously can have an equally detrimental effect on food webs based on the composition of those inputs.

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APPENDIX A: TABLES

Table 1. Comparison of nutritional quality and digestibility of Bt stalks, isogenic stalks, Bt leaves, and isogenic leaves.

	Bt		Isogenic	
	Stalks	Leaves	Stalks	Leaves
Crude protein	2.6%	4.5%	2.6%	4.9%
Lignin	1.2%	0.9%	1.1%	1.2%
Carbon	19.8%	17.9%	19.8%	20.6%
Nitrogen	0.4%	0.7%	0.4%	0.8%

APPENDIX B: FIGURES

Figure 1. Survival of *O. rusticus* exposed to Bt corn (white circle), isogenic corn (white square), Bt corn mixed with *P. occidentalis* (black square), isogenic corn mixed with *P. occidentalis* (black diamond), and *P. occidentalis* (black circle).

Figure 2. Mean $\pm$ SE weekly consumption of detritus by *O. rusticus* exposed to Bt corn, Bt mixed with *P. occidentalis*, isogenic corn, and isogenic corn mixed with *P. occidentalis* for eight weeks.

Figure 3. Percentage of *O. rusticus* that molted when exposed to Bt corn (white circle), isogenic corn (white square), Bt corn mixed with *P. occidentalis* (black square), isogenic corn mixed with *P. occidentalis* (black diamond), and *P. occidentalis* (black circle).