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BIOLOGICAL CONTROL OF THE BLACK CUTWORM, AGROTIS IPSILON (LEPIDOPTERA: NOCTUIDAE), AND ENDOPHYTE MEDIATED TRITROPHIC INTERACTIONS IN TURFGRASS

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ABSTRACT OF DISSERTATION

Andrea Jeanne Bixby-Brosi

The Graduate School

University of Kentucky

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Andrea Jeanne Bixby-Brosi
Lexington, Kentucky

Director: Dr. Daniel A. Potter, Professor of Entomology
Lexington, Kentucky

2011

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BIOLOGICAL CONTROL OF THE BLACK CUTWORM, AGROTIS IPSILON (LEPIDOPTERA: NOCTUIDAE), AND ENDOPHYTE MEDIATED TRITROPHIC INTERACTIONS IN TURFGRASS

Components of successful pest management programs must be complementary and not antagonistic. This project examined interactions between natural enemies of the black cutworm, Agrotis ipsilon (Hufnagel), an important turfgrass pest, and host plant resistance by endophytic grass.

Agrotis ipsilon nucleopolyhedrovirus (AgipMNPV) was examined as a bioinsecticide for controlling A. ipsilon in turfgrass. Fresh (1-week-old) AgipMNPV residues killed 76–86% of neonates hatching from eggs on golf course tees, however, residual control of implanted larvae lasted no more than a few weeks. Combinations of AgipMNPV with adjuvants, such as optical brightener and lignin, failed to accelerate or extend efficacy of the virus. AgipMNPV seems better suited for targeted control of early instars than for season-long control. Several applications per growing season would likely be needed to maintain high enough titers on turfgrass to effectively control cutworms.

The addition of a chitin synthesis inhibiting turfgrass fungicide failed to synergize AgipMNPV infectivity to A. ipsilon. Choice tests revealed the fungicide residues to be a mild feeding deterrent, the likely cause of slightly reduced mortality from virus infection seen in field trials. Combination applications in turfgrass might interfere with larval ingestion of a lethal virus dose, resulting in prolonged feeding in the field.

I examined how feeding on perennial ryegrass (Lolium perenne) with or without Neotyphodium lolii, its alkaloid-producing fungal endophyte, affects susceptibility of A. ipsilon to AgipMNPV. Feeding on endophytic grass neither compromises nor synergizes infectivity of AgipMNPV in the cutworm midgut. However, reduced consumption or avoidance of less-palatable endophytic grass could decrease ingestion of virus and rates of subsequent mortality in the field.
Host feeding on endophytic grass had differing effects on the tachinid fly, *Linnaemya comta*, a fast-developing solitary parasitoid, and the encyrtid wasp, *Copidosoma bakeri*, a slow-developing gregarious parasitoid. *L. comta* development did not appear to be affected when its host fed on endophytic grass; in contrast, *C. bakeri* suffered negative fitness effects. These results suggest that parasitoid life strategy and taxonomy play a role in endophyte mediated tritrophic interactions.

**KEYWORDS:** *Agrotis epsilon* nucleopolyhedrovirus, biological control, *Neotyphodium*, tritrophic interactions, chitin synthesis inhibitor
BIOLOGICAL CONTROL OF THE BLACK CUTWORM, *AGROTIS IPSILON* (LEPIDOPTERA: NOCTUIDAE), AND ENDOPHYTE MEDIATED TRITROPHIC INTERACTIONS IN TURFGRASS

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Introduction

Turfgrasses cover more than 20 million hectares of land in the United States including an estimated 60 million home and commercial lawns, about 17,000 golf courses, numerous parks, over 700,000 athletic fields, cemeteries, and other sites (National Turfgrass Initiative 2003). Aside from their aesthetic and recreational benefits, turfgrasses are important in preventing soil erosion, filtering water and runoff from urban areas, improving air quality, and provide safety and dust control along highways and airport runways (Beard 1994, National Turfgrass Initiative 2003). The calming and tranquilizing effects of cool green grass and other urban plantings where people live, work, and play is increasingly important to the mental health and physical well-being of residents in crowded urban and suburban areas (National Turfgrass Initiative 2003). Because of these benefits, turf management has become a multi-billion dollar per year industry in the United States.

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) is a major pest on golf course greens, tees, and fairways, and on sport fields in the United States and other temperate regions of the world where turfgrasses are grown (Potter 1998). Black cutworm larvae form silk lined burrows in the soil or thatch and emerge at night to feed on grass blades. Larvae are active for most of the night with the greatest activity between midnight and one hour before sunrise (Williamson and Potter 1997a). Early instars window-feed, mid-sized larvae crawl over the surface of the turf and chew down random grass blades, while large larvae confine their feeding to grass blades surrounding their burrow (Williamson and Potter 1997a). Cutworm damage, and the associated damage from foraging on cutworms by birds and other predators, causes
brown sunken pock marks, decreases aesthetic quality, and reduces smoothness, uniformity, and playability of putting surfaces.

In Kentucky there are three to four generations of *A. ipsilon* each year (Williamson 1996). The species apparently cannot overwinter in Kentucky or anywhere north of the transition zone (Potter 1998). Spring infestations begin about mid-March to early May as migratory adults arrive from overwintering sites in the southern states. Moths may be carried hundreds of kilometers in just a few nights by strong southerly winds (Showers et al. 1989). Eggs are laid on the tips of grass blades (Williamson and Potter 1997a). The last generation emerges from early September into late fall (Potter 1998).

Due to its multiple generations per year and low damage thresholds on golf courses, black cutworm often is the target for multiple insecticide applications each growing season (Potter 1998, Vittum et al. 1999, Williamson et al. 2011). Golf course putting greens receive more pesticides per unit area than any other turfgrass site (Smith and Tillotson 1993). Insecticides used to control black cutworm larvae on golf courses are mostly new chemistries, and are less toxic to non-target organisms than chemicals used in the past. However, some still have the potential to adversely affect pollinators, decomposers, and beneficial natural enemies (predators and parasitoids) that help keep cutworm and other pest populations in check (Potter 1998, Kunkel et al. 2001, Rogers and Potter 2003, Gels et al. 2002, Frank and Shrewsbury 2004, Held and Potter 2012).

Some insecticides have the potential to leach or run off into surface or ground water, and to impact aquatic organisms and wildlife (Culley et al. 1983, Shuman et al. 1983).
2000, Watschke et al. 2000). Insecticide use has also become a social concern in some communities that have mandated the use of only “organic” fertilizers and pesticides on turf. Initiatives towards environment stewardship in turfgrass include increasing numbers of sustainably or organically-managed golf courses, courses participating in environmental stewardship programs, e.g., the Audubon Cooperative Sanctuary certification program which promotes management practices that improve the quality of courses for wildlife (Dodson 2000, Anonymous 2006, Lyman et al. 2007), and The National Wildlife Federation program through which residential landscapes can earn certification as Backyard Habitats.

These issues have led to increased restrictions and loss of some insecticide registrations. For example, pyrethroids, which are widely used against surface feeding pests like the black cutworm, have relatively low mammalian and avian toxicity, but some are classified as restricted use because of toxicity to fish. The California Department of Pesticide Regulation recently placed pyrethroids into reevaluation (DPR 2006) because of their potential to harm aquatic systems. Numerous other examples exist (e.g., Racke 2000, Potter 2005, Bélair et al. 2010). The turf industry would benefit from a preventive biological control that would sustain itself in golf course habitats for potential season-long or even multi-year suppression of A. ipsilon.

Baculoviruses (family: Baculoviridae; genus: Nucleopolyhedroviruses), present a seemingly good alternative to broad-spectrum insecticides because of their efficacy, specificity, and safety to humans and other non-target organisms. Baculoviruses have been used to control insect pests in agricultural and forest settings (Cunningham 1995, Black et al. 1997, Moscardi 1999, Szewczyk et al. 2006, Erlandson 2008), but none have
been developed or marketed for use on turf. Prater et al. (2006) documented a natural
epizootic of Agrotis ipsilon nucleopolyhedrovirus (AgipMNPV) decimating populations
of the black cutworm on central Kentucky golf courses. In field trials in fairway-height
creeping bentgrass (Agrostis stolonifera L.), freshly sprayed residues of AgipMNPV gave
about 80% lethal infection of third or fourth instars after 1–4 day, and about 50% lethal
infection after 4 week. That groundwork suggests that AgipMNPV could provide short-
term and residual control of black cutworms in turfgrass settings (Prater et al. 2006).

Baculoviruses have limited host ranges, usually being restricted to one host
species or genus, with some exceptions (Moscardi 1999). They only infect larval feeding
stages where they form occlusion bodies (OBs), which are proteinaceous structures that
contain virus particles. The OB is the infectious stage of the virus and is important in
spreading the virus between hosts (Cory and Myers 2003). After ingestion, the
combination of the alkaline pH and proteases of the midgut dissolve OBs and release the
virions that then pass through the peritrophic membrane (Cory and Myers 2003). The
released virions fuse with the plasma membrane of the midgut columnar cells and move
to the nucleus to initiate infection (Cory and Myers 2003). Replicated virions use the
tracheal system as a conduit to reach the hemolymph and cause secondary infections in
other tissues (Engelhard et al. 1994). During infection the host larva is debilitated,
resulting in suppression of development, feeding and mobility, and experiences increased
vulnerability to predation (Moscardi 1999). The larval body tissue fills with millions of
OBs that are released into the environment or onto the substrate as the cadaver degrades
(Cory and Myers 2003). Diseased and dead larvae serve as inoculum for virus
transmission which may occur by rain and movement of arthropods on plants, or via predators or parasitoids (Moscardi 1999).

Limitations of baculovirus-based insecticides include UV irradiation and degradation of OBs and relatively slow speed of kill, especially of larger instars (Carruthers et al. 1988). One approach to improving baculovirus activity in the field is to use adjuvants such as optical brighteners and lignin. Optical brighteners have been used as fluorescent stains (Darken 1961) to protect entomopathogenic viruses, fungi, and nematodes from inactivation by solar radiation (Ignoffo and Batzer 1971, Shapiro 1992, Nickle and Shapiro 1994). Lignin, a natural plant polymer found in vascular plants and trees, is also an effective UV protectant (Tamez-Guerra et al. 2000, Arthurs et al. 2006, Peng and Argyropoulos 2007). Another approach is to facilitate infectivity of the virus itself. Chitin synthesis inhibitors have been shown to synergize baculoviruses and dramatically increase their activity by disrupting peritrophic membrane function (Arakawa 2002, 2003).

Turfgrass ecosystems contain a variety of predaceous and parasitic arthropods that may influence insect pest populations and are of particular interest to pest management and conservation biological control (Frank and Shrewsbury 2004). The black cutworm has several known parasitoids including the braconid wasps Meteorus rubens Nees (Caballero 1992, Awadallah et al. 1995, Zaki et al. 1997; 1995) and Apanteles ruficrus Hal, (Awadallah et al. 1995) and the tachinid fly Linnaemia comta Fallen (Rubink and Clement 1982). These natural enemies, however, have only been studied in corn ecosystems. Identification of black cutworm parasitoids in a turfgrass system will
provide a better understanding of how to conserve them for biological control purposes and how to recruit them to infested sites.

Several grasses (Poaceae) form symbiotic relationships with endophytic fungi, *Neotyphodium* spp., growing intercellularly within their leaf and stem tissues (Siegel et al. 1987; Schardl et al. 2004). The endophyte derives nutrients from its host plant while producing alkaloids that enhance resistance of the grass to herbivory (Breen 1994; Siegel and Bush 1996; Clay 1997; Schardl et al. 2004). *Neotyphodium lolii*, the endophyte associated with perennial ryegrass (*Lolium perenne* L.), produces three classes of alkaloids: ergot alkaloids, peramine, and lolitrems, contributing in varying degrees to protect from herbivores (Siegel and Bush 1996; Bush et al. 1997; Schardl et al. 2006; Potter et al. 2008). Because endophyte-infected grasses are deterrent or toxic to non-adapted herbivores, including certain grass-feeding insects (Siegel et al. 1987; Clay 1991; Breen 1994), establishing or overseeding them on lawns, sport fields, or golf courses can be useful in suppressing certain leaf- and stem feeding pests (e.g., Breen 1994, Richmond et al. 2000). Some endophyte-adapted species such as the black cutworm, *A. ipsilon*, however, can feed and complete their development on endophytic grass although such larvae develop more slowly than those that feed only on endophyte-free grass (Williamson and Potter 1997b; Kunkel and Grewal 2003; Potter et al. 2008).

Fungal endophytes may also affect the natural enemies of insect herbivores feeding on virus infected grasses. For parasitoids, such multitrophic effects are mediated by both sequestration and transmission of mycotoxins through food webs and by small size and poor nutritional quality of hosts feeding on endophytic grasses. Effects range from reduced growth, survival, or fecundity of individual species (e.g., Barker and
Addison 1996; 1997, Bultman et al. 1997) to altered energy transfer from plants to higher trophic levels affecting whole parasitoid communities (Omacini et al. 2001). Such effects have not been previously studied in turfgrass systems. Similar interactions can occur between endophytic grasses, herbivores, and entomopathogens. For example, *A. ipsilon* and fall armyworm, *Spodoptera frugiperda* (Smith), showed reduced susceptibility to nematodes when feeding on endophytic grass (Kunkel and Grewal 2003, Richmond et al. 2004), evidently because endophyte-produced alkaloids are toxic to the nematodes’ symbiotic bacterium (Kunkel et al. 2004, Richmond and Bigelow 2009).

This dissertation focuses on biological control of the black cutworm in turfgrass by the use of baculovirus, *Agip*MNPV, and parasitoids. The compatibility of these approaches with host plant resistance by endophytic grass is also investigated.
Objectives

One of the main goals of my study was to evaluate AgipMNPV as a bio-insecticide for season-long or multi-year suppression of black cutworm in large plot experiments under realistic golf course conditions. Adjuvants and optical brighteners that may increase the persistence of the virus in the field and in the insect itself were evaluated. The compatibility of endophyte associated host plant resistance and biological control by AgipMNPV and black cutworm parasitoids was investigated.

My specific objectives were to:

1. Determine infectivity and persistence of AgipMNPV to black cutworm in sand-based and soil-based putting greens and fairway height creeping bentgrass.
2. Evaluate AgipMNPV as a bio-insecticide for season-long and multi-year preventive control of black cutworm on golf courses.
3. Investigate adjuvants that may increase cutworm susceptibility to AgipMNPV.
4. Determine if a chitin synthesis inhibiting fungicide increases black cutworm susceptibility to AgipMNPV.
5. Investigate compatibility of endophytic turfgrasses with biological control of black cutworms by AgipMNPV.
6. Examine how feeding on endophytic grass affects the development of two parasitoids with different life strategies developing in A. ipsilon.

This dissertation is organized into four chapters, each formatted as a refereed paper containing its own Introduction, Materials and Methods, Results, and Discussion sections. Following Chapter 4, the section “General Conclusions” will not attempt to
summarize each chapter, but rather present some perspective and to discuss future directions and implications. All references are listed together in a single section at the end of the dissertation.
Chapter One

Evaluating a Naturally-Occurring Baculovirus for Extended Biological Control of the Black Cutworm (Lepidoptera: Noctuidae) in Golf Course Habitats

Introduction

Microbial products currently constitute < 0.1% of insecticides used on golf courses, lawns, and sport fields (Grewal 1999). Although modern chemical insecticides labeled for turfgrass are less broadly toxic than many used in the past, some still have the potential to intoxicate pollinators and other beneficial invertebrates, leach or run off into surface or ground water, or impact aquatic organisms (Potter 1998). Over-reliance on insecticides also has led to resistance in certain turfgrass insect pests (Ramoutar et al. 2009). Because of those issues, and especially in response to societal concerns and increased restrictions on pesticides, it is imperative for the turfgrass industry to move toward sustainable, reduced-risk tactics for pest management (Potter 2005, 2008).

Turfgrass insect pests are naturally infected by various pathogens (Cranshaw and Klein 1994, Klein 1995), but of those, only *Paenibacillus popilliae* (Dutky), causal agent of milky disease in Japanese beetle (*Popillia japonica* Newman) grubs, and entomopathogenic nematodes presently are marketed in the United States (Potter 2005). *Bacillus thuringiensis* Berliner (Bt) products are labeled against grass-feeding caterpillars but rarely used by the turf industry because of their short residual and poor activity against larger larvae (Klein 1995). A product with the fungus *Beauveria bassiana* (Balsamo) was briefly marketed in the 1990s but withdrawn due to inconsistent performance. Hurdles to commercialization of microbial turfgrass insecticides include
their narrow spectrum of activity, relatively high cost, short shelf life, problems with in vitro production, and competition from highly effective synthetic insecticides (Grewal 1999).

Baculoviruses (family Baculoviridae), because of their virulence, speed-of-kill, and persistence on protected plant parts and in soil (Cory et al. 1997), seem suited for controlling lepidopteran turfgrass pests. Baculoviruses have been used to control insect pests in agricultural and forest settings (Cunningham 1995, Black et al. 1997, Moscardi 1999), but none have been developed or marketed for use on turf. Prater et al. (2006) documented a natural epizootic of *Agrotis ipsilon* nucleopolyhedrovirus (*Agip*MNPV) decimating populations of the black cutworm, *Agrotis ipsilon* (Hufnagel), on central Kentucky golf courses. In field trials in fairway-height creeping bentgrass, freshly-sprayed residues of *Agip*MNPV gave about 80% lethal infection of third or fourth instars after 1–4 d, and about 50% lethal infection after 4 week. That groundwork suggests that *Agip*MNPV could provide short-term and residual control of black cutworms in turfgrass settings (Prater et al. 2006).

*Agrotis ipsilon* is a logical target for a baculovirus insecticide because it is a nearly worldwide pest of golf course putting greens and tees, as well as sport fields (Williamson and Potter 1997a,b; Potter 1998). The larvae are active at night, chewing down the grass surrounding their burrows in thatch and soil and causing brown pock marks that reduce smoothness and uniformity of playing surfaces (Potter 1998). Birds foraging on the caterpillars pull up tufts of grass, compounding the injury. Golf course superintendents often apply insecticides several times each growing season for cutworm control. Viral occlusion bodies can persist on leaf undersides, in protected plant parts,
and in the underlying soil (Cory et al. 1997, Peng et al. 1999). Turfgrass is a perennial system with dense foliage and thatch, so once a reservoir of virus is established, it seemingly would be protected from UV degradation and weather. It is not known, however, if the frequent irrigation, mowing, and chemical inputs typical on golf courses will affect the virus efficacy on tees or putting greens under play.

Optical brighteners used as fluorescent stains for microbes (Darken 1961) have been used to protect entomopathogenic viruses, fungi, and nematodes from inactivation by solar radiation (Ignoffo and Batzer 1971, Shapiro 1992, Nickle and Shapiro 1994). Lignin, a natural plant polymer found in vascular plants and trees, is also an effective UV protectant (Tamez-Guerra et al. 2000, Arthurs et al. 2006, Peng and Argyropoulos 2007). Fluorescent brighteners can also synergize viruses, facilitating their arrival at the host midgut epithelial cells, their initial target site, by disrupting the peritrophic membrane (Wang and Granados 2000, Mukawa et al. 2003, Okuno et al. 2003). Most studies showing the benefits of adjuvants were done in laboratory or greenhouse settings (e.g., Martin 2004, Mukawa et al. 2003, Shapiro 1992), and none have involved turfgrass.

This study evaluated AgipMNPV as a bio-insecticide for short-term and extended control of black cutworms in turfgrass settings representative of golf course habitats and on whole golf tees under actual play. Our working hypothesis was that preventively applying the virus would deposit enough infective occlusion bodies in the thatch and soil to suppress successive generations of A. ipsilon during the growing season. Adjuvants intended to enhance viral performance and protect the virus from UV degradation were also tested in combination with field applications of AgipMNPV. New information on
the parasitoid complex impacting *A. ipsilon* populations on Kentucky golf courses was obtained from recovery of sentinel larvae in the various experiments.

**Methods and Materials**

**Insects and Virus.** *Agrotis ipsilon* eggs and larvae from a colony maintained on soybean-based diet were shipped from a commercial insectary (Benzon, Carlisle, PA) via overnight mail and transferred to our assays within a few hours of arrival. The *AgipMNPV* isolate used was originally obtained from naturally-infected, late-instar *A. ipsilon* from central Kentucky golf courses (Prater et al. 2006). Frozen infected caterpillars were macerated in 0.1% sodium dodecyl sulfate (SDS) for 10 min, filtered through five layers of cheese cloth, and then centrifuged at 900 × g for 10 min. The pellet contents were resuspended in 0.5% SDS and centrifuged again. Re-suspension and centrifugation were repeated with 0.5M NaCl with the final suspension in distilled water. Sodium azide was added at 0.02% concentration to prevent bacterial growth. This purified occlusion bodies (OB) suspension was stored at 4 °C. OB concentrations were determined using a phase-contrast microscope and a Neubauer bright-line hemocytometer (Fisher, Pittsburgh, PA).

**Small-Plot Field Evaluations in Golf Course-type Settings.** Field trials evaluating *AgipMNPV* against *A. ipsilon* were conducted on three types of sites representative of golf course settings at the University of Kentucky’s Turfgrass Research Center (UKTRC), Spindletop Farm, near Lexington. Independent replicated trials were concurrently run on a soil-based (push up) putting green, a sand-based putting green, and in an adjacent stand of fairway-height grass. The turfgrass at all three sites was
‘Penncross’ creeping bentgrass, *Agrostis stolonifera* L., on a Maury silt loam (fine, mixed, mesic typic Paleudalf) with a pH of 6.3. The push-up green and fairway stand were on homogeneous, original soil where no sand topdressing had been applied. The sand-based green was constructed with a 90 - 10% sand content root zone mix according to United States Golf Association Method guidelines (Beard 2002). Both greens were mowed at 4.0 mm five times per week and irrigated from a permanent sprinkler system to prevent visible drought stress. Nitrogen fertilizer (urea: 46-0-0) was applied to all sites in September, October, and November at 0.48 kg actual N per 100 m² per application. Fungicides were applied curatively for control of fungal diseases, and dithiopyr (0.07 kg AI/ha) was applied for crabgrass control. The fairway-type turf was mowed at 1.6 cm three times per week and irrigated as necessary to prevent drought stress.

Six pairs of treated and untreated 1 m² plots were marked on each site. Purified virus suspension was applied at $10 \times 10^8$ OB per treated plot in 162 ml of distilled water using a hand sprayer (Solo, Newport News, VA) on 11 September 2007. Three passes were made in alternating directions to ensure even coverage. Light post-treatment irrigation (162 ml per plot) was applied to move virus residues to lower grass blades, stems, thatch, and upper soil. Twenty, third instar *A. ipsilon* were introduced onto each plot 1 week and 6 weeks after application (18 September and 26 October) and again on 13 May 2008 to determine if the virus had remained infective in the thatch layer. Larvae were confined in circular metal enclosures (39.0 cm diam × 10.2 cm height) driven 2 cm into the ground. We used different quadrants of each plot for the successive infestations. Enclosures were covered with 0.64-cm mesh wire hardware cloth to prevent bird predation. Grass was not mowed while cutworms and enclosures were in the plots.
Surviving larvae were recovered after 4 days by using a soap flush consisting of 1.3 ml lemon-scented Joy dishwashing detergent (Proctor & Gamble, Cincinnati, OH) per liter of water (Potter 1998). Larvae were rinsed with distilled water as soon as they surfaced, placed in individual capped 30-ml cups with soybean-based noctuid diet (Blanco et al. 2009) and monitored until death or pupation. Death due to viral infection was verified by examining hemolymph for viral OB using a phase contrast microscope.

**Residual Activity on a Closely-Mowed Putting Green versus Surrounds.** On golf courses, *A. ipsilon* developing from eggs laid in peripheral areas (surrounds) often crawl onto putting greens as late instars (Williamson and Potter 1997a). Because higher-mowed turf seemingly would be a less harsh environment for virus longevity, applying *Agip*MNPV to surrounds might provide longer-lasting control than treating only greens (Prater et al. 2006). That hypothesis was tested on a sand-based creeping bentgrass green at the UKTRC, maintained as described above, and in the irrigated surrounds consisting of a mixture of creeping bentgrass, clover, and tall fescue mowed at 1.6 cm three times per week. Six pairs of treated and untreated 1 m$^2$ plots were marked on both the green and about 2 m into the adjacent surrounds. Purified virus suspension was applied at $10 \times 10^9$ OB per treated plot in 162 ml of distilled water using a hand sprayer. Twenty, early third instars were introduced onto each plot 4 days after application, followed by groups of 20 second instars at 14 and 36 days after application. Larvae were confined in metal enclosures, as described earlier, with different plot quadrates used for each challenge. Surviving larvae were recovered 4 days post-infestation using a soap disclosing solution, rinsed and placed individually in cups with artificial diet, and death from viral infection was assessed as described above.
Residual Activity With or Without Adjuvants. An experiment initiated in August 2008 tested whether increased protection and residual activity is provided to AgipMNPV by an optical brightener (Blankophor P167- Bayer Corp., Pittsburgh, PA) a lignin (Polyfon, MeadWestvaco, Charleston, SC), or a combination thereof. The study site consisted of fairway-height creeping bentgrass maintained as described above. Virus suspensions were prepared as described earlier, with adjuvants added at 1% of the final volume of solution. Combination treatments contained 1% of each adjuvant. Treatments included brightener & virus; lignin & virus; brightener, lignin & virus, and virus alone, plus untreated controls. Virus solutions (40 ml per plot) were applied with a hand sprayer to small (0.25m²) plots at 10 × 10⁹ OB m⁻². Four replicates of each treatment were applied 5 weeks, 3 weeks, and 3 days before larvae were introduced, except the brightener, lignin & virus combination, which was only applied at 5 and 3 weeks before challenge because available virus was limited at the time. Metal enclosures, as described earlier, were driven into the turf, and all plots were simultaneously challenged with 20 second instars on 18 September 2008. The larvae were left to feed for 5 days before being recovered via soap flush, rinsed, placed individually in 30 ml cups with soybean diet, and monitored until death or pupation. Death from virus infection was assessed as before.

Residual Efficacy of AgipNMPV on Golf Course Tees under Play. Field tests were conducted on whole tees and surrounding areas at two central Kentucky golf courses (University Club Golf Course, Lexington; Cherry Blossom Golf Course, Georgetown, KY) in 2008. Six holes (i.e., the composite unit made up of the tees, fairway, and green) were chosen on each course and two tees per hole were selected for
use. Both courses have 4–5 oval or rectangular tees per hole; only back and front tees were used because they receive less play. All tees were composed of creeping bentgrass, which was mowed at 1.3 cm three times per week, and irrigated to prevent visible drought stress.

One tee of each hole, as well as a 2 m buffer of surrounding higher-mowed turf, was sprayed with virus \((10 \times 10^8 \text{ OB m}^{-2})\) on each course. The \textit{Agip}MNPV suspension needed to treat the 12 total tees and surrounds required about 16,000 virus-killed late-instar \textit{A. ipsilon} (8,000 per golf course) which were cultured in the lab in winter 2007–08 and frozen until enough virus was produced. Virus suspensions were delivered using an 18.9 l backpack sprayer (Solo, Newport News, VA) at a spray volume of 162 ml/m\(^2\) between 1800–2200 hours. \textit{A. ipsilon} crawl onto tees from surrounds (Williamson and Potter 1997b), so treating a buffer zone may reduce populations. Treating in the evening may reduce UV light degradation. Six similarly-sized tees on the same holes served as controls on each course. Tees were treated in early May and maintained by the golf course greenskeepers under their normal management regimes, except that no surface insecticides were applied.

Virus residual efficacy was determined by sampling naturally-occurring \textit{A. ipsilon} populations and by inoculating the tees with eggs or larvae. The former were sampled by soap flush once per month on three 1 m\(^2\) areas of each tee. In addition, each tee was inoculated with 20 second instars and 150 eggs three times (mid-May, mid-June, early August 2008). Neonates (120 per tee) also were implanted once, 2 months after application on 27 July and 2 August at Cherry Blossom and University Club respectively. Second instars were confined in 39-cm diameter metal enclosures covered with wire
mesh, as described earlier. Eggs and neonates were confined in white PVC cylinders (10.0 cm diam × 10.2 cm height) driven 2 cm into the ground to prevent escape and predation by non-flying insects. Different areas of each tee were used on each sample or inoculation date, and those dates were on alternate weeks for the two golf courses because of the large amount of time required to treat, inoculate, and sample the 12 tees on each course.

Surviving larvae from the egg and larval challenges were recovered by soap flush 10 days post-introduction. All naturally occurring or inoculated larvae recovered in the samples were held on diet until death or pupation, which was followed by evaluation of incidence of virus infection, evaluated by microscopic examination of hemolymph as described earlier.

**Parasitoids.** Cutworms recovered from all experiments were held until death or pupation. Any larva showing signs of parasitism was watched closely until adult parasitoids had emerged. Wasps and flies were identified by C. A. Boring (University of Kentucky) and E. R. Hoebeke (Cornell University), respectively. Voucher specimens are deposited in the University of Kentucky Insect Collection.

**Statistical Analyses.** Because all assays were done in the field, recovery of larvae following egg or larval challenges was variable. In most cases, 5–20 larvae were recovered per plot. Analyses for numbers of normal or virus-infected larvae recovered were based on counts so all replicates were included. Lethal virus infection in the small plot trials simulating different golf course settings, the comparison of putting green turf versus surrounds, and the trials on whole tees under play was analyzed using one-tailed paired t-tests \( (H_1: \text{treated} > \text{control}) \). Percentage data were normalized by arcsine square-
root transformation before analysis, and those few plots from which fewer than five larvae were recovered were treated as missing values so as not to calculate percentages on inadequate sample sizes.

For evaluation of efficacy with or without spray adjuvants, percentages of larvae dying from virus were examined by weighted factorial analysis of variance (ANOVA) for main effects and interaction of treatments and virus residue age. Percentage of larvae dying from virus within 3 days after recovery from the field also was analyzed to test the hypothesis that adjuvants would accelerate lethal infection. Within each residue age, a weighted two-way ANOVA and one-sided Dunnett’s tests were used to compare treatments in the absence of the control, and to compare treatments versus control, respectively. The brightener/lignin combination was excluded from ANOVA factorial analyses because it had not been applied on all dates, creating an unbalanced experimental design. It was, however, included for analyses of infection in larval cohorts exposed to 3- or 5-weeks old residues. Statistix 8 (Analytical Software 2008) was used for all statistical analyses except for weighted ANOVAs, where SAS (Statistical analysis software 9.1) was used. Percentages were arcsine square-root transformed for analysis. All data are reported as original (non-transformed) means ± standard error (SE).
Results

Small-plot Field Evaluations in Golf Course-type Settings. One-week old AgipNMPV residues resulted in 50–60% lethal infection when challenged with third instars (Figure 1). Infection rates from the 1-week challenge were higher in treated than in untreated plots regardless of type of site (soil-based green: $t = 2.8$, df = 5, $P = 0.02$; sand-based green: $t = 3.6$, df = 5, $P < 0.01$; fairway height turf: $t = 2.87$, df = 5, $P = 0.02$). However, there was almost no infection on any of the sites when the treated turf was challenged with third instars 6 weeks after application, or again the following May. Hemolymph smears revealed only 11 virus-infected larvae (4.9%) among those recovered from treated plots after 6 weeks, and only one virus-killed larva the following May.

Residual Activity on Closely-Mowed Putting Green and Surrounds.

Percentages of larvae killed by 3-day old virus residues did not significantly differ between paired plots on the sand-based creeping bentgrass putting green surface or higher-mowed mixed-grass surrounds (59 ± 10.4 versus 50 ± 4%, respectively; $t = -2.57$, df = 3, $P = 0.8$). Infection in treated plots was lower in the 2-week challenge (15 ± 5 versus 9 ± 5% lethal infection, respectively), again with no difference between putting green plots and surrounds ($t = -0.69$, df = 2, $P = 0.7$). Mortality from exposure to 3-day old residues was higher in treated plots than in controls in both settings (putting green: 59 ± 10.4 versus 7 ± 5.0% infection, respectively; $t = 3.3$; df = 5; $P = 0.01$; surrounds: 50 ± 4 versus 0 ± 0% infection, respectively; $t = 19.37$; df = 4; $P < 0.01$). Infection rates were similarly low in treated and untreated plots on the green and in the surrounds at 2 weeks after application (putting green: 15 ± 4.5 versus 2.5 ± 1.6%, respectively; $t = 1.8$; df = 4;
surrounds: 8.6 ± 5.6 versus 0.8 ± 0.8% infection, respectively; t = 0.68; df = 4; 
P = 0.3). No larval death due to virus infection occurred at any site when larvae were 
exposed to 4-week old AgipMNPV residues.

**Residual Activity With or Without Adjuvants.** Overall infection was 86, 60, 
and 20% at 4 days, 3 weeks, and 5 weeks after application, respectively, with significant 
decline over time (Figure 2; F = 18.6; df = 2, 22; P < 0.01). The adjuvants, however, did 
not synergize or prolong infectivity (F = 0.04; df = 3, 22; P = 0.95). Four-day old 
residues of all treatments gave significantly higher rates of lethal infection than occurred 
in controls. Virus alone was the only treatment that provided significantly higher lethal 
infection 3 weeks after application when compared to the control (Dunnett’s test, P < 
0.05), however, its efficacy was similar to all other treatments (F = 0.46; df = 3, 8; P = 
0.72). Five weeks after application, infection levels were low, highly variable, and did 
not significantly differ in any of the treatments (F = 0.09; df = 3, 7; P = 0.97).

Percentage of larvae dying from virus within 3 days after recovery from the field 
decreased as virus residues aged (F = 28.8; df = 2, 22; P < 0.01), but did not differ 
between treatments (F = 0.56; df = 2, 22; P = 0.62), nor was there date by treatment 
interaction (F = 0.71; df = 4, 22; P = 0.60) (Figure 2).

**Residual Efficacy of AgipMNPV on Golf Course Tees under Play.** Fresh (10- 
day old) virus residues reduced numbers of healthy (i.e., not lethally infected by virus) 
larvae recovered from tees that had been inoculated with eggs in mid-May by 76% (t = - 
2.3; df = 5; P = 0.03) and 82% (t = -1.9; df = 5; P = 0.05) at University Club and Cherry 
Blossom golf courses, respectively (Figure 3). Similarly, 41% fewer healthy larvae (t = - 
2.36; df = 5; P = 0.03) were recovered from treated than from non-treated tees at
University Club in June, 10 days after the second inoculation with eggs at 4 week after treatment; however there was no significant reduction at Cherry Blossom (Figure 3). Numbers of non-infected larvae recovered following the third inoculation with eggs, 12 week after treatment, did not differ between treated and non-treated tees at University Club (11.5 ± 3.2, 3 ± 1.9, respectively; \( t = 2.9; \ df = 5; \ P = 0.98 \)), and in fact, were somewhat higher on the treated than on the non-treated tees. Very few larvae were recovered following the same challenge at Cherry Blossom. No significant treatment effect was evident when the tees were challenged with cohorts of neonates two months after treatment at University Club (9.5 ± 3.1, 17.8 ± 5.5 healthy cutworms recovered from treated versus non-treated tees, respectively; \( t = -1.17; \ df = 5; \ P = 0.15 \)) or at Cherry Blossom (3.2 ± 1.7, 6.5 ± 3.0, respectively; \( t = -0.82; \ df = 5; \ P = 0.22 \)).

Lethal infection of introduced second instars was significantly higher in treated plots at 1 week (\( t = 7.3; \ df = 5; \ P \leq 0.01 \)) and 4 week (\( t = 2.83; \ df = 5; \ P = 0.01 \)) after virus application at University Club (Figure 4). At Cherry Blossom, however, percent lethal infection of second instars did not differ between treated and non-treated tees at 1 week (15 ± 5.4, 7 ± 3.1, respectively; \( t = 0.6; \ df = 3; \ P = 0.29 \)) or 4 weeks (9.7 ± 5.8, 10 ± 10, respectively; \( t = 1.5; \ df = 2; \ P = 0.13 \)) after application. Natural infestation of the tees by \( A. \, ipsilon \) was quite low, with only a few feral larvae recovered by soap drench sampling, so those data were not analyzed.

**Parasitism.** Parasitoids emerged from 40 of the 164 neonate larvae (24%) that had been introduced onto golf tees at University Club in June and recovered by soap flush after 10 days. Of those, 30 were killed by the tachinid fly, *Linnaemya comta* (Fallen), seven by braconid wasps, and the other three were parasitized by *Copidosoma*
bakeri (Howard), a polyembryonic encyrtid wasp. At Cherry Blossom, 20 of the 65 larvae (35%) recovered following the neonate introductions were parasitized, 10 by braconid wasps (two Meteorus spp. and one Microplitis sp.), six by C. bakeri, and four by L. comta. C. bakeri parasitizes eggs of A. ipsilon (Saeki et al. 2009) so its recovery in samples of larvae presumed to have originated from larval inoculations indicates that some larvae recovered in those samples were from the natural population. Seven of the 87 larvae recovered from the tees at Cherry Blossom following the 13 July egg inoculation were parasitized, six by braconid wasps and one by C. bakeri. At University Club, only one of the 87 cutworms recovered from the 10 July egg inoculation was parasitized by a braconid, but 22% (19 of 87) of those recovered following the 28 August egg inoculation of the same tees were parasitized, 15 by braconids, three by C. bakeri, and one by L. comta. No parasitoids emerged from other inoculations at either golf course.

**Discussion**

Baculoviruses could realistically compete with chemical insecticides for managing A. ipsilon or other grass-feeding caterpillars on golf courses or sport fields if one application provided extended suppression once the virus became established in the turfgrass (Prater et al. 2006, Potter 2008). Potential markets for AgipMNPV or other biological insecticides include sustainably or organically-managed golf courses, courses participating in environmental stewardship programs, e.g., as wildlife sanctuaries (Dodson 2000, Anonymous 2006, Lyman et al. 2007), sites where surface runoff of chemicals into ponds or streams is a concern (Watschke et al. 2000), and sport fields or other sites where the use of chemical insecticides is prohibited or poses undue liability.
Turfgrasses typically have a thatch layer composed of living and dead stems, stolons, and roots and partially decomposed organic matter that accumulates between the living plants and soil (Hurto et al. 1980). Thatch binds and retains pesticide residues (Niemczyk and Chapman 1987, Dell et al. 1994, Spieszlski et al. 1994), so we speculated it would retain a virus reservoir that would induce lethal infections for many weeks or months. Virus occlusion bodies can also persist on the underside of leaves, in protected plant parts, and in the topsoil (Cory et al. 1997). The hypothesis that higher-mowed grass would protect the virus longer from UV degradation was not, however, supported by infection rates in larval challenges. Fresh virus residues killed a high percentage of *A. ipsilon* in all heights of turf, but efficacy lasted only a few weeks regardless of whether the residues were on or around greens, on tees, or in fairway-height grass. The frequent mowing, clipping removal, and irrigation on golf courses doubtless contribute to movement of virus out of the turfgrass and, together with UV degradation, account for loss of residual activity within a few weeks.

Commercially-produced baculoviruses have been used to manage insect pests in agricultural and forest settings (Cunningham 1995, Black et al. 1997, Moscardi 1999, Szewcyk et al. 2006) and in some cases one application has provided season-long or multi-year control (Zelanzny et al. 1992, Fuxa 1991). Examples of this include inoculative releases of a non-occluded virus of the rhinoceros beetle, *Oryctes rhinoceros* L., in coconut palms (Zelanzny et al. 1992), and releases of the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, baculovirus in soybean,(Fuxa 1991). Our trials on whole creeping bentgrass tees are the first to evaluate a baculovirus for season-long control of a pest on golf courses under actual maintenance and play. Although 1-week old virus
residues reduced larval populations resulting from implanted eggs by 76–82%, elevated infection of implanted larvae lasted no more than a few weeks.

Combining insect-pathogenic viruses with optical brighteners (Ignoffo and Batzer 1971, Shapiro 1992) or lignin (Tamez-Guerra et al. 2000, Arthurs et al. 2006, Peng and Argyropoulos 2007) may screen them from UV degradation and enhance their longevity. In this study, however, such adjuvants failed to accelerate or extend efficacy of *Agip*MNPV against *A. ipsilon* in fairway-height creeping bentgrass. Frequent mowing and clipping removal of golf course fairways, tees and greens would soon remove viral occlusion bodies deposited on grass blades, so residual virus would be mostly on lower portions of the plants or in the thatch or upper soil. Any screening by adjuvants might be less evident in a relatively dense turfgrass canopy than in more exposed settings. Stilbene optical brighteners also bind to the chitin in the caterpillar midgut, disrupting peritrophic membrane formation and increasing larval susceptibility to baculovirus infection (Wang and Granados 2000). Thus their use as adjuvants can provide comparable infection of target pests at lower virus application rates (Shapiro and Robertson 1992, Shapiro 1992, Boughton et al. 2001). The relatively high application rate used in our field trial, at which virus alone gave 80–90% infection for as long as 3 weeks, may have masked any short-term synergism that might have been apparent at a lower rate.

Most of the previous studies documenting synergism of a baculovirus by optical brighteners were in systems where the plant canopy offered significant protection from sunlight (Webb et al. 1994, Zou and Young 1996). Benefits of adding a brightener might be less evident in more exposed turfgrass systems, since some brighteners themselves can
be degraded within a few days of application (Vail et al. 1999). Boughton et al (2001), for example, found that the optical brightener M2R, which reduced the LD$_{50}$ of AgipMNPV to *A. ipsilon* in the laboratory, failed to enhance its efficacy against the same pest in greenhouse- or field-grown corn.

In caterpillars, a defense mechanism known as midgut cell sloughing, triggered by virus-challenged midgut epithelial cells, becomes increasingly developed in later instars (Hoover et al. 1999). Neonate *A. ipsilon* are especially vulnerable to AgipMNPV whereas later instars must ingest higher dosages to become lethally infected (Prater et al. 2006). Black cutworm moths deposit eggs on grass blades in turfgrass settings (Williamson and Potter 1997a) so neonates would be exposed to virus residues and ideally are killed before reaching destructive size. First instars are highly susceptible to insect predation (López and Potter 2000) and difficult to sample by soap drench and recover from turf field plots, so second or third instars were used for most trials herein. Mortality from virus would likely have been higher had neonates been used, a supposition supported by the 76–82% reduction in larval population when the virus-sprayed tees were inoculated with eggs. Lethal infection of the second and third instars probably also would have been higher had the larvae been left to feed longer in the treated turf, rather than being sampled, brought back, and transferred to virus-free diet for assessment after a few days.

Golf putting greens receive more pesticides per unit area than any other turfgrass sites (Smith and Tillotson 1993), so they are a focal point for concerns about insecticide leaching, surface runoff, and potential exposure to golfers. The game, however, requires smooth uniform playing surfaces, especially on greens, so many golf course
superintendents treat for cutworms multiple times per growing season. In the northeastern United States, another key pest, the annual bluegrass weevil (Listronotus maculicolis Lietz; Coleoptera: Curculionidae), is increasingly resistant to pyrethroids (Ramoutar et al. 2009), which likely has been exacerbated by reliance on the same chemical class for cutworm control. By diversifying options for cutworm control, an AgipMNPV-based bio-insecticide could be useful in resistance management of L. maculicolis and other turf pests (e.g., billbugs, chinch bugs) having similarly limited dispersal and out-crossing capability.

Little is known about the identity of black cutworm parasitoids in turfgrass habitats or how much they contribute to biological control. Although parasitism was not the focus of this study, parasitoids emerged from 24 and 31% of the cutworms recovered following neonate larval introductions on tees of the two golf courses in June, and from 22% of larvae recovered from egg inoculation sites at University Club in August. Of those, Meteorus spp. and Microplitis sp. oviposit into larvae (Bixby and Potter, unpublished data), whereas C. bakeri is a polyembryonic encyrtid that oviposits in the host egg (Saeki et al. 2009). Females of the tachinid L. comta are attracted to host frass and larviposit near the cutworm burrow; the planidial maggot then burrows into the host larva (Clement et al. 1982, Rubink and Clement 1982, Bixby, unpublished data). Clearly those parasitoids, and likely others, contribute to biological control of A. ipsilon in turfgrass, so conservation of their benefits is another reason why a selective, baculovirus-based insecticide would be useful for managing cutworms on golf courses.

In summary, while it was hoped that AgipMNPV could provide season-long control of A. ipsilon in golf course settings, our results suggest it may be better suited for
targeted knock-down of early instars than for season-long residual control. Golf courses are a severe environment for entomopathogens. Daily or frequent mowing and clipping removal, irrigation, and other intensive management practices are not conducive to maintaining lethal titers of baculoviruses on grass foliage, so multiple applications per growing season may be required to manage cutworms or other grass-feeding caterpillars on such high-profile sites as putting greens. Nonetheless, for our trials we were limited to spraying a self-made crude AgipMNPV suspension. Better control possibly could be gained by selecting for AgipMNPV strains having higher virulence, or by formulating the virus with synergists or performance enhancing adjuvants other than those tested herein. As with most entomopathogens, commercial success of AgipMNPV would be facilitated by advances in in-vitro production methodology allowing the virus to be produced more economically and in greater amounts (Kompier et al. 1988, Weiss et al. 1994, Black et al. 1997). Despite those hurdles, turf provides a strong potential market for biological insecticides, and efforts to develop AgipMNPV or other baculoviruses for sustainable golf course management are warranted.
Figure 1. Mean (± SE) percentage lethal infection of 3rd instar A. ipsilon following 4-day exposure to 1-week old residues of AgipNMPV applied at $10 \times 10^8$ occlusion bodies m$^{-2}$ in three different golf course-type habitats. Lethal infection was significantly greater in treated than in untreated plots in all settings (soil-based green: $t = 2.8$, df = 5, $P = 0.02$; sand-based green: $t = 3.6$, df = 5, $P < 0.01$; fairway height turf: $t = 2.87$, df = 5, $P = 0.02$).
Figure 2. Residual infectivity of *Agip*MNPV applied at $10 \times 10^9$ occlusion bodies m$^{-2}$ either alone or in combination with 1% optical brightener, lignin, or both. Data are means (± SE). The legend denotes the age of residues on 18 Sept when each plot was challenged with 20 second instars for 5 days. The treatment with both adjuvants was not tested (NT) with 4-day old residues because stocks of virus were limited at that time. Adjuvants did not synergize or prolong infectivity (see text).
Figure 3. Mean (± SE) numbers of viable second and third instars (excluding ones lethally infected by virus) recovered 10 days after inoculating golf course tees under play with *A. ipsilon* eggs. There were six treated and six non-treated tees on each golf course. Separate cohorts of eggs were introduced 1 and 4 weeks after treated tees had been sprayed with *Agip*MNPV (10 × 10⁹ occlusion bodies m⁻²). Asterisks denote challenges in which there were fewer viable larvae on the treated tees (one-tailed paired *t*-tests; *P* ≤ 0.05).
Figure 4. Mean (± SE) percentage lethal infection by AgipMNPV in cohorts of second-instar A. ipsilon introduced onto treated and untreated golf course tees at 1, 4, or 12 weeks after the virus was applied. These data are from University Club only. Asterisks denote challenges in which virus residues provided significantly higher infection than occurred on the non-treated tees (one-tailed paired t-tests; $P \leq 0.05$).
Chapter Two

Influence of Endophyte (*Neotyphodium lolii*) Infection of Perennial Ryegrass on Susceptibility of the Black Cutworm (*Lepidoptera: Noctuidae*) to a Baculovirus

Introduction

Plant secondary chemicals can alter susceptibility of insect herbivores to naturally encountered pathogens as well as to microbial insecticides applied for biological control (Jones 1984, Berenbaum and Zangerl 1988, Cory and Hoover 2006). Caterpillar mortality, for example, can differ by as much as 50-fold depending on the species of host plant upon which baculoviruses or *Bacillus thuringiensis* subsp. *kurstaki* (BTk) are consumed (e.g., Keating et al. 1988, Duffey et al. 1995, Hoover et al. 1998, Farrar and Ridgeway 2000, Kouassi et al. 2001, Ali et al. 2004). Phytochemicals can deactivate pathogens on the leaf surface (Young et al. 1977, Duetting et al. 2003), bind to or deactivate virus occlusion bodies (OBs) in the larval midgut (Felton and Duffey 1990), or reduce cell permissiveness to infection (Foster et al. 1992, Ali et al. 1998, Cory and Hoover 2006). Alternatively, they may synergize pathogens by disrupting the peritrophic matrix and allowing easier passage to the hemocoel (Plymale et al. 2008) or by stressing the host and impairing normal immune function (Cory and Hoover 2006). By modifying an herbivore’s foraging or feeding, phytochemicals can alter the likelihood of it encountering pathogens or of acquiring a lethal pathogen dose (Baverstock et al. 2005, Dwyer et al. 2005).
Several grasses (Poaceae) form symbiotic relationships with endophytic fungi, *Neotyphodium* spp., growing intercellularly within their leaf and stem tissues (Siegel et al. 1987, Schardl et al. 2004). The endophyte derives nutrients from its host plant while producing alkaloids that enhance resistance of the grass to herbivory (Breen 1994, Siegel and Bush 1996, Clay 1997, Schardl et al. 2004). Endophyte-infected grasses are deterrent or toxic to numerous insect species (Siegel et al. 1987, Clay 1991, Breen 1994). *Neotyphodium lolii*, the endophyte associated with perennial ryegrass (*Lolium perenne* L.), produces three classes of alkaloids: ergot alkaloids, peramine, and lolitrems, contributing in varying degrees to protection from herbivores (Siegel and Bush 1996, Bush et al. 1997, Schardl et al. 2006, Potter et al. 2008). The black cutworm *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae), however, can feed and complete its development on endophytic perennial ryegrass although such larvae develop more slowly than those that feed only on endophyte-free grass (Williamson and Potter 1997a, Kunkel and Grewal 2003, Potter et al. 2008).

Fungal endophytes may also affect the natural enemies of insect herbivores feeding on virus infected grasses. For parasitoids, such multitrophic effects are mediated by both sequestration and transmission of mycotoxins through food webs and by small size and poor nutritional quality of hosts feeding on endophytic grasses. Effects range from reduced growth, survival, or fecundity of individual species (e.g., Barker and Addison 1996; 1997, Bultman et al. 1997) to altered energy transfer from plants to higher trophic levels affecting whole parasitoid communities (Omacini et al. 2001).

Similar interactions can occur between endophytic grasses, herbivores, and entomopathogens. For example, *A. ipsilon* feeding on endophytic (E+) perennial ryegrass
or tall fescue, *Schedonorus phoenix* (Scop.), were less susceptible to entomopathogenic nematodes, *Steinernema carpocapsae* Weiser, than were larvae fed endophyte-free (E−) grass, evidently because endophyte-produced alkaloids are toxic to the nematodes’ symbiotic bacterium (Kunkel and Grewal 2003, Kunkel et al. 2004, Richmond and Bigelow 2009). Fall armyworms *Spodoptera frugiperda* (Smith), too, showed reduced susceptibility to nematodes when feeding on E+ perennial ryegrass (Richmond et al. 2004). Such endophyte-tolerant caterpillars may sequester the endophytic alkaloids as an acquired defense against nematode-induced septicemia (Richmond and Biglow 2009).

Evidence for endophyte effects on nematode-induced mortality of root-feeding scarabaeid grubs is equivocal; one study (Grewal et al. 1995) suggested that grubs feeding on E+ fescue were more susceptible to nematodes, whereas another similar study (Koppenhöfer and Fuzy 2003) showed no interaction. Infectivity of milky disease bacteria, *Paenibacillus popilliae* (Dutky), to larval Japanese beetle, *Popillia japonica* Newman, was similar in E+ or E− perennial ryegrass (Walston et al. 2001).

*Agrotis ipsilon* is a major pest of turfgrasses on golf courses and sport fields where its feeding and associated bird predation reduces the aesthetics, smoothness, and uniformity of playing surfaces (Potter 1998). Recently, a naturally occurring baculovirus (*Agrotis ipsilon* nucleopolyhedrovirus; family Baculoviridae; AgipMNPV) was shown to have promise as a microbial insecticide for controlling *A. ipsilon* in turf (Prater et al. 2006). No previous studies have examined how feeding on E+ or E− grasses may interact with caterpillars’ susceptibility to a baculovirus. Because use of E+ perennial ryegrass and tall fescue is a sustainable approach to managing billbugs, chinch bugs, aphids, and other susceptible insect pests that feed on turfgrass leaves and stems (e.g.,
Breen 1993; 1994, Murphy et al. 1993, Richmond et al. 2000, Richmond and Shetlar 2000), it is important to determine if such grasses are compatible with baculovirus infection of *A. ipsilon* and other relatively less endophyte-susceptible lepidopteran pests that occur in such grasses. E+ grasses also occur in pastures where synergism or antagonism with naturally-occurring baculoviruses could dampen or accentuate outbreaks of grass-feeding caterpillars.

This study examined tritrophic interactions between E+ or E− perennial ryegrass and susceptibility of *A. ipsilon* to AgipMNPV. In addition to mortality, we evaluated speed of kill, which for other pathogens can be affected by phytochemistry (Raymond et al. 2002, Cory and Hoover 2006). Separate experiments were done with larvae allowed to feed *ad libitum* on virus-treated grass, and in which they received one-time doses to control for possible differences in consumption associated with palatability of the grasses.

**Materials and Methods**

**Insects and Virus.** *Agrotis ipsilon* eggs and larvae were obtained from a commercial insectary (Benson, Carlisle, PA, USA) where they had been maintained on soybean-based diet. They were shipped in cups of artificial diet by overnight mail and transferred to our assays within a few hours of arrival. The AgipMNPV isolate used in all experiments was originally obtained from naturally infected late instar *A. ipsilon* from central Kentucky golf courses (Prater et al. 2006). Frozen infected caterpillars were macerated in 0.1% sodium dodecyl sulfate (SDS) for 10 min and filtered through five layers of cheese cloth. OBs were then centrifuged at 900 × g for 10 min. The pellet was resuspended in 0.5% SDS and centrifuged again. Re-suspension and centrifugation were
repeated with 0.5M NaCl with the final suspension in distilled water. Sodium azide was added at 0.02% concentration to prevent bacterial growth. This purified OB suspension was stored at 4°C. OB concentrations were determined using a phase-contrast microscope and a Neubauer bright-line hemocytometer (Fisher, Pittsburg, PA).

**Plants and Endophyte.** ‘Rosalin’ perennial ryegrass, with or without endophyte (N. lolii), was planted in 11.4 × 11.4 cm plastic pots (3 mg seed/cm²) in a glasshouse. Seed was obtained from C. L. Schardl (Department of Plant Pathology, University of Kentucky). Endophyte infection, determined by staining representative tillers with an aniline blue solution (Shelby and Dalrymple 1987) was 88% and 0% for E+ or E− grass, respectively. Alkaloid concentrations in the potted E+ grass were not determined in this study; however, tillers grown from the same batch of seeds were previously reported (Potter et al. 2008) to have the following amounts (µg/g dry weight) in their grass blades: ergovaline (1.24 ± 0.12), ergine (0.12 ± 0.02), chanoclavine (1.43 ± 0.19), 6,7-secolysergine (0.77 ± 0.08) and peramine (135 ± 58).

Grasses were maintained under 14-hour photoperiod with supplemental lighting from 1000 W sodium vapor bulbs unless ambient light was ≥450 µmol m⁻² s⁻¹. Day and night temperatures were set at 22°C and 18°C, respectively. The potting mix consisted of 3:1 Pro-Mix BX (Premier Horticulture, Quakertown, PA) and autoclaved topsoil. Plants were fertilized with Peters 20-10-20 Peat-Lite formula (Scotts, Marysville, OH), trimmed to 5 cm, and watered as needed.

**Infectivity of Virus Consumed ad libitum on Treated E+ or E− Grass.** Ten weeks after grasses were established, five third-instar A. ipsilon were added to each pot
and allowed to feed for 3 days before virus treatments were applied. Two pots (10 larvae) constituted an experimental unit, and there were 10 replicates (100 total larvae) per treatment. Sheer nylon cages made from women’s knee high hose supported by wooden plant stakes were fitted over each pot to prevent larval escape. Virus suspensions were applied at concentrations of $5 \times 10^9$, $5 \times 10^8$, $5 \times 10^7$, and $5 \times 10^6$ OBs per experimental unit in 20 ml of distilled water. Cages were removed, the grass within each pot was treated with one of four rates of virus using a hand held sprayer (Solo, Newport News, VA), and cages then were replaced. The pots were segregated during spraying and neoprene gloves were worn during handling to minimize cross-contamination. Control pots were not sprayed. Treatments were arranged in a randomized complete block in the greenhouse.

Larvae were allowed to feed in the pots of grass for 10 days and then recovered by pulling the grass plugs, examining the soil, roots, thatch, and grass, and removing all larvae found. The grass plug then was placed back into the pot, and any remaining larvae were extracted using a soap disclosing solution (5 ml lemon-scented Ultra Joy [Proctor & Gamble, Cincinnati, OH] dishwashing soap per 3.7 L of water). Those few remaining larvae brought up by the soap were rinsed with fresh water as soon as they surfaced. All recovered larvae were weighed, determined to instar based on head capsule width, placed individually in 37 ml rearing cups (Dart Container, Mason, MI) with soybean based diet (Benson, Carlisle, PA), and monitored until death or pupation. Days until death and instar at death were recorded. Death due to viral infection was verified by examining blood for OBs using a phase contrast microscope at 400 × magnification.
Interaction Between Endophyte and Virus Consumed as a One-time Dose.

Neonate *A. ipsilon* were reared in the laboratory (25 ± 0.5º C; 14:10 (L:D) h photoperiod) on fresh grass clippings from E+ or E− plants from egg hatch until they reached the second instar (about 12 days). They were then placed individually into empty 30 ml plastic diet cups, and larval weight and instar were recorded. Aqueous suspensions of virus were prepared by mixing virus stocks with distilled water and blue food coloring. A hemocytometer was used to determine the concentration of OBs in each suspension. Larvae were starved for 4−5 hours, and then a 2µl droplet of virus suspension containing 0, 50, 1525, or 3025 OBs per droplet was placed by pipette on the bottom of each cup with the larva. Larvae were given 1 hour to ingest the droplet and only those that consumed the entire droplet were used. The experiment contained five replicates of 10 fully-dosed larvae per treatment. Immediately after ingestion of the droplet, clippings of either E+ or E− grass were placed in each diet cup. Grass was replaced every 2 days with fresh clippings, and larvae were monitored until death or pupation. Weight and instar of survivors were recorded 10 days after administering virus. Death due to viral infection was verified by examining blood for OBs using a phase contrast microscope, and days until death was also recorded.

**Statistical Analysis.** Larval weights, percentage mortality from virus, instar at death, days until death, and other variables were analyzed by a $2 \times 5$ (*ad libitum* feeding experiment) or a $2 \times 4$ (single-dose feeding experiment) factorial analysis of variance (ANOVA) for main effects and interaction of virus rate and endophyte. For the single-dose feeding experiment, a three way repeated-measures ANOVA also was conducted on cumulative percentage mortality. Fixed factors were endophyte and virus rate (between-
subjects factors) and time after exposure to virus (within-subjects factor), with repeated measure (mortalities) on the time factor, as mortalities were recorded on groups of larvae within the same replicates over time. Percentage data were normalized by arcsine square-root transformation for analysis but are reported as non-transformed values. Effect of virus rate was analyzed by polynomial contrasts for significance of linear or quadratic trends. Control data were excluded when analyzing days until death and instar at death because most larvae in the control groups survived to pupation making sample sizes for death-related parameters very small. Statistix 8 (Analytical Software 2008) was used for all statistical analyses. All data are reported as means ± standard error (SE).

Results

Infectivity of Virus Consumed ad libitum on Treated E+ or E− Grass.

Cutworms fed E+ grass had significantly lower average 10-day weights than larvae fed E− grass (Table 1). Larval weight decreased with increasing rate of virus, and mortality also increased, with significant linear and quadratic effects for rate. Although E+ fed cutworms were smaller than comparably-aged larvae feeding on E− grass, there was similarly high mortality due to virus infection regardless of grass type (Table 1). When the controls (no virus applied) were excluded, the effect of rate on mortality was still significant ($F = 3.5; \text{df} = 3, 63; P = 0.02$), but the percentage of larvae that died from virus was slightly higher on E− than on E+ grass ($F = 3.9; \text{df} = 1, 18; P = 0.05$). Infected larvae also died more rapidly when feeding on E− grass (Table 1), suggesting that those cohorts ingested a lethal dose of virus more quickly than did their counterparts feeding on E+ grass.
Interaction Between Endophyte and Virus Consumed as a One-time Dose.

Larvae again gained significantly less weight on E+ than on E− grass. Percentage mortality from virus increased linearly with increasing dose, but with a similar response for larvae fed E+ and E− grasses (Table 2). Virus dose had no significant effect on weight gain (Table 2). Larvae died more rapidly at higher virus doses; however, unlike in the previous trial in which virus-sprayed grasses were consumed ad libitum, there was no difference between days until death for larvae fed E+ and E− grass ($F = 0.8; \text{df} = 1, 18; P = 0.4$) when the virus was droplet fed as a single dose. There was no interaction between endophyte and virus dose for any variable examined.

Repeated measures ANOVA on cumulative percentage mortality indicated significant effects for rate ($F = 25.8; \text{df} = 3, 16; P < 0.01$), time ($F = 73.9; \text{df} = 5, 80; P < 0.01$), and rate × time interaction ($F = 10.2; \text{df} = 15, 80; P < 0.01$). Thus, lethal infection increased with increasing virus rate but larvae died at similar rates regardless of grass type (Figure 5). Other interactions were not significant.

Discussion

Planting or overseeding with endophytic turfgrasses can suppress various leaf- and stem-feeding insect pests (Breen 1993; 1994, Murphy et al. 1993, Richmond et al. 2000), but the value of this approach for lawns, sport fields, or golf courses could be compromised if relatively endophyte-tolerant pests (e.g., *A. ipsilon* or *S. frugiperda*) are rendered less susceptible to their own natural enemies. This study, evidently the first to examine how feeding on E+ grass affects susceptibility of a caterpillar to baculovirus, indicates that biological control of *A. ipsilon* by AgipMNPV is compatible with use of E+
perennial ryegrass. There was, however, delayed and slightly reduced mortality from *AgipNMPV* when larvae fed *ad libitum* on virus-sprayed E+ as opposed to E− ryegrass.

Black cutworms prefer E− perennial ryegrass and avoid feeding on E+ grass when given a choice (Williamson and Potter 1997a, Potter et al. 2008). In both of our experiments, larvae developed faster on E− than on E+ grass, but despite their larger size, infected larvae died more quickly when feeding *ad libitum* on the E− grass after both sets of grasses were sprayed with virus. No such effect was seen when the virus was consumed as a single dose, supporting the hypothesis that in the *ad libitum* feeding trial, larvae consumed lethal amounts of virus more quickly on the relatively more palatable E− grass. Reduced consumption or avoidance of virus-contaminated E+ grass could affect the likelihood that *A. ipsilon* will encounter or ingest a lethal dose of virus in the field.

Numerous studies indicate that phytochemicals such as phenolics can increase, or more often decrease, mortality of host insects from baculoviruses, but the mechanisms for such changes often are unclear (Cory and Hoover 2006). Alkaline plant exudates at the phylloplane can deactivate baculoviruses via premature dissolution of OBs (Corey and Hoover 2006). Inactivation of *Heliothis* NPV applied to cotton, *Gossypium hirsutum*, for example, was attributed to high ion concentrations and alkalinity of cotton dew; however, deactivation did not occur on soybean, *Glycine max* (Young et al. 1977). Many phytochemicals appear to interfere with pathogen infection in the host gut (Corey and Hoover 2006). Phytochemicals can be directly antagonistic to baculoviruses in the larval midgut. In one such study, quinones from tomato, *Lycopersicon esculentum*, reduced
*Heliothis zea* susceptibility to a baculovirus by binding to and reducing the digestibility and solubility of OBs, thereby impairing the release of infective virions (Felton and Duffey 1990). Midgut sloughing, which enables caterpillars to clear primary virus infections, can be accelerated by plant secondary chemistry (Hoover et al. 1998; 2000).

We cannot rule out the possibility that partial deactivation of viral OBs by endophytic alkaloids at the phylloplane contributed to the delay in mortality when *A. epsilon* fed *ad libitum* on virus-treated grass. However, this delay did not occur when larvae were reared on E+ or E− grasses until 4 hours before ingesting the AgipMNPV as a single dose. While it is possible that the endophyte-produced alkaloids consumed before dosing had already passed through the digestive tract during the 4 hour starvation period, the larvae immediately resumed feeding on their respective grasses after being droplet-fed the virus, so E+ or E− grass was present in the crop and midgut at the same time as AgipMNPV. This suggests that the aforementioned delay in virus-induced mortality and the slight decrease in incidence of lethal infection when virus was consumed on treated E+ grass probably cannot be attributed to deactivation in the larval gut.

Constitutive or induced plant secondary chemicals can modify the physiology and growth of insect hosts affecting their susceptibility to infection (Duffey et al. 1995, Ali et al. 1998). Induced changes in wounded tomato foliage resulted in stunting of *Heliothis zea*, accompanied by 50% greater mortality from NPV infection than occurred on non-wounded foliage (Ali et al. 1998). Smaller stressed larvae often require lower pathogen doses to initiate infection (Duffey et al. 1995, Cory and Hoover 2006), and if such insects also eat more to compensate for poor plant quality, they would further increase their risk of ingesting a lethal pathogen dose. *A. epsilon* are developmentally stunted when reared
on E+ grass (Williamson and Potter 1997a, Potter et al. 2008), and early instars are especially susceptible to AgipMNPV (Prater et al. 2006), so synergism between endophyte and virus seemed a reasonable possibility. Our results, however, failed to support that hypothesis.

This study suggests that microbial control of A. ipsilon by naturally-occurring or applied baculoviruses should be compatible with use of E+ grasses. Reduced feeding on or migration of larvae from E+ grasses, however, could somewhat reduce the impact of a virus in field settings. Many cutworms on golf courses develop in higher-mowed turf surrounding creeping bentgrass putting greens, moving onto the greens as later instars (Williamson and Potter 1997b). Grasses surrounding greens typically consist of perennial ryegrass, tall fescue, Kentucky bluegrass, or mixtures of those grasses. Treating the surrounds with AgipMNPV to reduce reservoir populations of A. ipsilon, a suggested management strategy (Prater et al. 2006), would be less effective if use of E+ grasses in such areas caused cutworms to migrate from the treated areas onto the greens before having ingested a lethal dose. Increased emigration from pure or mixed stands of E+ grasses has been shown for other species of grass-feeding insects (Richmond and Shetlar 1999; 2000). Alternatively, use of E+ tall fescue or perennial ryegrass in surrounds might have net positive results for pest management if the slower development and smaller size of A. ipsilon feeding on such grasses exposed them to greater mortality from predators and parasitoids that take a heavy toll on the early instars (López and Potter 2000, Bixby unpublished data). Endophytes affect both the physiology and behavior of grass-feeding insects, so the outcome of using combinations of E+ grasses and baculoviruses for pest management may be difficult to predict.
Table 1. Effects of endophyte (*Neotyphodium lolii*) and rate of application of *Agip*MNPV on larval weight and infectivity of the virus to *A. ipsilon*, and absence of interaction between those factors.

<table>
<thead>
<tr>
<th>Rate (OBs)</th>
<th>Endophyte</th>
<th>No. larvae recovered(^a)</th>
<th>10-day weight (mg)</th>
<th>% mortality</th>
<th>Instar at death</th>
<th>Days to death</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>8.1 ± 0.5</td>
<td>153 ± 8</td>
<td>3 ± 2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.1 ± 0.6</td>
<td>123 ± 6</td>
<td>8 ± 3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5×10(^6)</td>
<td>-</td>
<td>6.9 ± 0.5</td>
<td>144 ± 8</td>
<td>79 ± 6</td>
<td>4.2 ± 0.1</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.4 ± 0.4</td>
<td>116 ± 6</td>
<td>63 ± 6</td>
<td>4.2 ± 0.2</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>5×10(^7)</td>
<td>-</td>
<td>6.8 ± 0.4</td>
<td>128 ± 9</td>
<td>69 ± 5</td>
<td>3.9 ± 0.1</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.9 ± 0.6</td>
<td>107 ± 6</td>
<td>52 ± 7</td>
<td>3.8 ± 0.1</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>5×10(^8)</td>
<td>-</td>
<td>6.0 ± 0.5</td>
<td>109 ± 6</td>
<td>65 ± 9</td>
<td>3.6 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.3 ± 0.5</td>
<td>89 ± 5</td>
<td>65 ± 10</td>
<td>3.4 ± 0.1</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>5×10(^9)</td>
<td>-</td>
<td>6.8 ± 0.5</td>
<td>87 ± 4</td>
<td>88 ± 3</td>
<td>3.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
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<tr>
<td></td>
<td>+</td>
<td>7.5 ± 0.3</td>
<td>82 ± 5</td>
<td>78 ± 4</td>
<td>3.2 ± 0.1</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

ANOVA (F values)\(^b\)

<table>
<thead>
<tr>
<th>Source</th>
<th>F values</th>
<th>df</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endophyte</td>
<td>0.8</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>Rate</td>
<td>2.7*</td>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>E × R</td>
<td>2.7*</td>
<td>1</td>
<td>0.05</td>
</tr>
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</table>

Polynomial contrasts (t)

<table>
<thead>
<tr>
<th>Component</th>
<th>t</th>
<th>df</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear trend</td>
<td>-1.2</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Quadratic trend</td>
<td>3.0*</td>
<td>1</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^a\)Out of 10 original larvae per replicate. All data are means (± SE).

\(^b\)For number of larvae recovered, % infected, and 10-day weights, degrees of freedom = 1, 4, 4, and 81 for Endophyte, Rate, E × R interaction, and Error, respectively. For remaining dependent variables, df = 1, 3, 3, and 63, respectively. * and ** denote significant at P < 0.05 and 0.01, respectively.
Table 2. Effects of endophyte *(Neotyphodium lolii)* and rate of a one-time dose of AgipMNPV on larval weight and infectivity of the virus to *A. ipsilon*, and absence of interaction between those factors.

<table>
<thead>
<tr>
<th>Dose (OB)</th>
<th>Endophyte</th>
<th>Starting weight (mg)</th>
<th>% mortality&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Weight diff&lt;sup&gt;b&lt;/sup&gt; (mg)</th>
<th>Instar diff&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>16 ± 2.0</td>
<td>4 ± 2</td>
<td>181 ± 8</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6 ± 0.4</td>
<td>2 ± 2</td>
<td>117 ± 11</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>20 ± 1.8</td>
<td>22 ± 6</td>
<td>167 ± 9</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6 ± 0.5</td>
<td>12 ± 5</td>
<td>104 ± 7</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>1525</td>
<td>-</td>
<td>25 ± 1.9</td>
<td>32 ± 10</td>
<td>205 ± 18</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4 ± 0.6</td>
<td>32 ± 4</td>
<td>105 ± 10</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>3025</td>
<td>-</td>
<td>18 ± 1.7</td>
<td>62 ± 7</td>
<td>190 ± 12</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5 ± 0.4</td>
<td>68 ± 10</td>
<td>81 ± 12</td>
<td>1.9 ± 0.1</td>
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ANOVA (*F* values)

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<th><em>F</em> value</th>
<th><em>P</em> value</th>
<th><em>F</em> value</th>
<th><em>P</em> value</th>
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<td>204**</td>
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<td>134**</td>
<td>2.8</td>
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<tr>
<td>Rate</td>
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<td>--</td>
<td>23.6**</td>
<td>1.9</td>
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<tr>
<td>E × R</td>
<td><em>(F</em> &lt;sub&gt;3, 28&lt;/sub&gt;)</td>
<td>--</td>
<td>0.7</td>
<td>2.6</td>
<td>0.8</td>
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Polynomial Contrasts (*t*)

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<th>Quadratic</th>
<th>Trend</th>
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<tr>
<td>--</td>
<td>8.0**</td>
<td>1.8</td>
</tr>
<tr>
<td>--</td>
<td>0.2</td>
<td>-1.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Of 10 larvae per replicate. <sup>b</sup>Difference between measurements taken before and 10 days after virus dose was administered. All data are means (± SE). * and ** denote significant at *P* < 0.05 and 0.01, respectively.
Figure 5. Mortality of *A. ipsilon* was observed 5, 7, 9, 10, 14, and 16 days after larvae had been droplet-fed a dose of *Agip*MNPV. Cumulative mean percent mortality refers to only those larvae that died from virus infection. Control, low, and high refer to dose treatments of 0, 50 and 3025 OBs, respectively. For clarity, responses to the medium dose of 1525 OB are not shown. Error bars (± SE) are included, but most are very small values and cannot be seen in the figure. Larvae died more rapidly at higher virus doses; however, there was little difference between days until death for larvae fed on E+ and E−grass (*F* = 0.8, df = 1, 18, *P* = 0.4).
Chapter Three

Can a Chitin Synthesis Inhibiting Turfgrass Fungicide Enhance Black Cutworm Susceptibility to a Baculovirus?

Introduction

Baculoviruses (family: *Baculoviridae*; genus: *Nucleopolyhedrovirus*), present a seemingly good alternative to broad-spectrum insecticides because of their efficacy, specificity, and safety to humans and other non-target organisms. They have been used worldwide to manage pests in various cropping systems and forests (Cunningham 1995, Black et al. 1997, Moscardi 1999, Szewczyk et al. 2006, Erlandson 2008). It is striking, however, given that > 400 insect species, mostly members of the orders Lepidoptera and Hymenoptera, have been reported as hosts for baculoviruses, how infrequently they are successfully used in integrated pest management programs (Tanda and Kaya 1993, Moscardi 1999, Lacey et al. 2001).

One of the limitations of baculovirus-based insecticides is their relatively slow speed of kill, especially of late instars (Bonning and Hammock 1996, Moscardi 1999, Szewczyk et al. 2006, Erlandson 2008). As larvae mature, they typically become less susceptible to virus infection and may continue to feed for several days after ingesting a lethal dose, so targeting early instars is necessary to avoid economic damage to plants (Hoover et al. 2000, Cory and Myers 2003, Prater et al. 2006). Most research to enhance the usefulness of baculoviruses has focused on using optical brighteners to protect them from degradation by ultraviolet light (Wang and Granados 2000, Mukawa et al. 2003, Okuno et al. 2003). Another approach is to increase the virulence of the virus itself. For
an insect to become infected, it must first ingest virus occlusion bodies (OBs) while feeding. After ingestion, the OBs release virions in the host midgut, which then must pass through the peritrophic membrane to cause a systemic virus infection (Cory and Myers 2003). This chitinous membrane is the insect’s first line of defense against a virus, so a compound that disrupts its function may help facilitate infection and increase speed of kill. Chitin synthesis inhibitors have been shown to synergize baculoviruses and dramatically increase their activity by disrupting peritrophic membrane function (Arakawa 2002; 2003).

Polyoxins are Streptomyces-derived antibiotics that inhibit fungal and insect chitin synthases (Hori et al. 1971, Cohen 1987, Decker et al. 1991). Polyoxin-d strongly affected peritrophic membranes in vitro in adult blowflies, Calliphora erythrocephala, by inhibiting chitin synthesis and by changing the fine structure of the membrane (Becker 1980). Nucleopolydorovirus (NPV)-susceptibility was increased in larvae of the silkworm, Bombyx mori, when commercially available polyoxin fungicidal agents were incorporated into the insect’s artificial diet (Arakawa 2002; 2003). Enhanced biological activity of Spodoptera litura NPV by a chitin synthesis inhibiting compound was attributed to obvious ruptures on the outer surfaces of the peritrophic membrane, which potentially facilitated the passage of virions through the peritrophic membrane (Hui-Fang et al. 2007). These compounds have been validated as synergists in laboratory experiments but have not been tested in the field.

The black cutworm, A. ipsilon, is nearly a worldwide pest of golf course putting greens and tees, as well as sport fields and various garden crops (Williamson and Potter 1997a, Potter 1998). In turf, the night-active larvae chew down the grass surrounding
their burrows, causing brown pock marks that reduce smoothness and uniformity of playing surfaces (Potter 1998). Prater et al. (2006), documented a natural epizootic of Agrotis ipsilon multicapsid nucleopolyhedrovirus (AgipMNPV) decimating black cutworm populations on Kentucky golf courses, established dose-mortality relationships, and demonstrated that a sprayed viral suspension can provide short-term control in the field (Prater et al. 2006). When sprayed suspensions of AgipMNPV were evaluated for season-long control of black cutworm on creeping bentgrass (Agrostis stolonifera L.) golf course tees under actual maintenance and play, 1-week-old virus residues reduced larval populations resulting from introduced eggs by 76–82%. Residual control, however, lasted no more than a few weeks (Bixby-Brosi and Potter 2010). AgipMNPV quickly controls young larvae, but larger late instars require higher dosages and continue to feed for several days before being killed (Prater et al. 2006, Bixby-Brosi and Potter 2010). Combinations of AgipMNPV with adjuvants, such as optical brightener and lignin, failed to accelerate or extend efficacy of the virus against A. ipsilon in the field (Bixby-Brosi and Potter 2010). Even if they had worked, such adjuvants likely would be too expensive to use in synergizing virus applications targeting grass-feeding caterpillars on golf courses or sports fields.

If baculovirus efficacy could be enhanced by something already being used in the turf or crop system, land managers would incur no additional cost. For example, fungicides containing the active ingredient polyoxin-d are already being used, sometimes several times per season, to control turfgrass diseases such as brown patch, Rhizoctonia solani Kühn An overlapping application of polyoxin-d fungicide and baculovirus would be a practical combination in golf course settings, because fungal diseases and cutworm
infestations often occur on the same tees, greens, and other highly maintained sites. The purpose of this study was to determine if the combined use of a chitin synthesis inhibiting substance, polyoxin-d, can enhance or synergize AgipMNPV activity against A. ipsilon in turfgrass.

**Materials and Methods**

**Insects, Virus, and Fungicide.** *Agrotis ipsilon* eggs and larvae were obtained from a commercial insectary (Benzon, Carlisle, PA, USA) where they had been maintained on soybean-based diet. They were shipped in cups of diet by overnight mail and transferred to our assays within a few hours of arrival. The AgipMNPV isolate used in all experiments was originally obtained from naturally infected late instar *A. ipsilon* from central Kentucky golf courses (Prater et al. 2006). Frozen infected caterpillars were macerated in 0.1% sodium dodecyl sulfate (SDS) for 10 min and filtered through five layers of cheese cloth. Virus OBs were then centrifuged at 900 × g for 10 min. The pellet was resuspended in 0.5% SDS and centrifuged again. Resuspension and centrifugation were repeated with 0.5M NaCl with the final suspension in distilled water. Sodium azide was added at 0.02% concentration to prevent bacterial growth. This purified OB suspension was stored at 4°C. OB concentrations were determined using a phase-contrast microscope and a Neubauer bright-line hemocytometer (Fisher, Pittsburgh, PA).

The polyoxin formulation evaluated as a synergist for AgipMNPV was Endorse® Wettable Powder Fungicide (Arysta LifeScience, Cary, NC), containing 2.5% active ingredient polyoxin-d zinc salt (equivalent to 2.2% polyoxorim and 0.3% metallic zinc),
Zinc 5-[[2-amino-5-O-(aminocarbonyl)-2-deoxy-L-xylonoylamino]-1-(5-carboxy-3,4-dihydro-2,4-dioxo-1(2H)-pyrimidinyl)-1,5-dideoxy-D-allofuranuronate. Endorse® is a group 19 fungicide, and is labeled for controlling fungal diseases on golf courses, residential lawns, parks, and commercial and institutional grounds. The wettable powder was dissolved in distilled water for all applications.

**Evaluating Virus/fungicide Combinations in Small Field Plots.** An experiment initiated in July 2010 tested whether increased activity is provided to *Agip* MNPV residues by the fungicide. The trial was conducted in a stand of ‘L-93’ creeping bentgrass on a Maury silt loam (fine, mixed, mesic typic Paleudalf; pH = 6) at the University of Kentucky’s Turfgrass Research Center (UKTRC), Spindletop Farm, near Lexington, KY. The turfgrass, representative of a golf course fairway, was mowed at 1.6 cm three times per week, irrigated as necessary to prevent drought stress, and fertilized in September, October, and November at 0.48 kg actual N per 100 m² per application from urea (46–0–0). Fungicides (non-poloxin) had been applied curatively, as needed, for control of fungal diseases, but were not used for at least 4 weeks before our trials.

Individual plots were 0.5 m², with 1 m² buffers, and arranged in a randomized complete block with six replicates of each treatment. Virus suspensions were prepared as described above. Treatments included high, medium, and low rates of virus (5×10⁸, 5×10⁷, and 5×10⁶ OB m⁻²) with and without fungicide, fungicide alone and an untreated control. Fungicide treatments were at a high label rate for golf course fairways [1.2 g (product) m⁻²]; virus rates were based on previous field experiments (Prater et al. 2006, Bixby-Brosi and Potter 2010). Larvae were confined in circular metal enclosures (39.0
cm diameter, 10.2 cm height) which were twisted and pressed to seat their lower edge about 1 cm into the ground. Each solution was dissolved in 50 ml of water and applied using a hand pump sprayer inserted into a 50 ml plastic vial. The area inside each enclosure (0.12 m²) was treated, and larvae were introduced as soon as the residues had dried.

Twenty, third instar *A. ipsilon* were introduced into each of the metal enclosures, which were then covered with 0.64-cm mesh wire hardware cloth to prevent bird predation. Grass was not mowed while cutworms and enclosures were in the plots. Surviving larvae were recovered after 4 days by using a soap flush consisting of 1.3 ml of lemon-scented dishwashing detergent (Joy®, Proctor & Gamble, Cincinnati, OH) per liter of water (Potter 1998). Larvae were rinsed with distilled water as soon as they surfaced, placed in individual capped 30-ml cups with soybean based noctuid diet (Blanco et al. 2009), held at 25°C, and monitored until death or pupation. Death due to viral infection was verified by examining blood for viral OBs by using a phase contrast microscope at 400 × magnification.

**Testing for Direct Insecticidal Effects of Fungicide.** The soap drench brought up relatively few cutworms from fungicide-treated plots in the above experiment, suggesting there had been a disproportionately high number of escapes from those enclosures, or mortality from the fungicide itself. Therefore, a follow-up trial was conducted at the same field site to determine if the fungicide alone reduced cutworm survival. Treatments included high and low rates of fungicide (1.2 and 0.6 g m⁻²) and an untreated control. Plots were again 0.5 m² with a 1 m² buffer, and set up in a randomized complete block design with six replicates of each treatment. The experiment was carried
out as described above; however, the metal enclosures were driven more deeply (3 cm) into the turf to ensure that larvae could not escape by burrowing beneath their edges.

**Testing Fungicide/virus Synergism; Greenhouse Trials.** In August 2010, creeping bentgrass cores (15.2 cm diameter, 6.5 cm deep) were harvested with an oversized golf course cup cutter from the aforementioned creeping bentgrass stand. Grass cores were placed in pots with a small amount of potting mix below and around them to help maintain moisture. The potting mix consisted of 3:1 Pro-Mix BX (Premier Horticulture, Quakertown, PA) and autoclaved topsoil. Plants were watered as needed. The turfgrass was maintained in a glass house under 14-hour photoperiod with supplemental lighting from 1000W sodium vapor bulbs unless ambient light was ≥450 l mol m$^{-2}$s$^{-1}$, and watered as needed to maintain vigor. Day and night temperatures were set at 22 and 18°C, respectively.

The treatments (virus/fungicide combinations) included high, medium, and low rates of virus (5 x 10$^9$, 5 x 10$^6$, and 5 x 10$^3$ OB m$^{-2}$) with or without fungicide at high, medium, and low rates (2.1 g m$^{-2}$, 0.21 g m$^{-2}$, and 0.012 g m$^{-2}$), plus an untreated control. Each solution was dissolved in 50 ml of water and applied using a separate hand pump sprayer inserted into a disposable tube containing the treatment combination. Six replicates of each treatment were arranged on greenhouse benches in a randomized complete block design. Treatments dried for 20 min before third instar *A. ipsilon* (12 per pot) were introduced into pots. Larvae were allowed to feed on treated grasses for 24 hours. Cutworms were recovered by removing the grass plugs from their containers and examining the soil, roots, thatch, and grass. The grass plug then was placed back into the pot, and those few remaining larvae were extracted using a soap disclosing solution and
immediately rinsed with fresh water to remove soap as soon as they surfaced. All larvae were placed individually in 30-ml rearing cups with artificial diet and monitored until death or pupation, as above. Days until death were recorded. Death due to viral infection was verified by examining blood for OBs using a phase contrast microscope.

The above experiment was repeated to determine how varying the duration of exposure by feeding cutworms might affect virus synergism by the fungicide. Two virus rates \(1 \times 10^8\) and \(5 \times 10^8\) OB m\(^{-2}\) and one fungicide rate \(2.1\) g m\(^{-2}\) were applied alone and in combination, plus an untreated control. Cohorts of five replicates per treatment were set up in a randomized complete block design to be sampled at three different times (after 1, 2, and 4 days of feeding and exposure).

**Fungicide Effects on Consumption of Treated Grass.** Feeding preference of neonates and third instars was compared between fungicide treated and untreated grass to try to reconcile results from the field and greenhouse experiments. More specifically, the hypothesis was tested that reduced consumption of fungicide-treated grass might interfere with cutworm ingestion of a lethal virus dose, thus resulting in lower infection rates. Creeping bentgrass cores were collected from the UKTRC on 13 September and maintained in a glass house as described above. Grass clippings were cut into 2.5 cm sections. The clippings were treated with the label rate of fungicide \((2.1\) g m\(^{-2}\)) by dipping them into the mixed fungicide solution for 5 sec and then allowing the residues to air dry. Three treated and three untreated clippings were placed in an alternating, spoke-like arrangement on a moistened filter paper in the bottom of a polystyrene petri dish (90 mm × 15 mm). Ten neonates were placed in the center of each dish before replacing the lid. For the no-choice tests, one treated or untreated grass blade and one neonate were
placed in each arena. There were 20 replicates for each test. Larvae were left to feed in the dark for 17 hours at room temperature (about 22°C). The total area of leaf tissue consumed of each treatment was visually estimated to the nearest 10% by two independent observers whose ratings were averaged, and the number of larvae actively feeding was also scored for each dish and treatment.

The trials were repeated with third instars, using larger arenas (styrofoam bowls, 115 mm × 50 mm). Grass blades were held in place on moistened filter paper using insect pins to prevent them from being scattered by the larvae. A single larva was added to each bowl; bowls were then capped with plastic wrap, covered with another styrofoam bowl, and placed in a dark growth chamber (27°C). The percentage of each grass blade that had been consumed was visually estimated, as above, at 1, 4, and 18 hours.

**Statistical Analysis.** Larval recovery and weights, percentage mortality from virus, and other variables were analyzed by a 2 × 4 (small plot field experiment) or a 4 × 4 (greenhouse experiment) factorial analysis of variance (ANOVA) for main effects and interaction of fungicide and virus rate (weighted ANOVA was used for field experiment percentages). Effect of virus rate was analyzed by polynomial contrasts for significance of linear or quadratic trends. A three way repeated-measures ANOVA also was conducted on cumulative percentage mortality for greenhouse experiments. Fixed factors were fungicide rate and virus rate (between-subjects factors) and time after exposure to treatments (within-subjects factor), with repeated measure (mortalities) on the time factor, as mortalities were recorded on groups of larvae within the same replicates over time. Dunnett’s tests were performed to compare virus mortalities in control groups (virus alone) to fungicide/virus combinations. The percentage of fungicide-treated or
untreated leaf tissue consumed in the choice and no-choice tests was compared by Wilcoxon signed-rank tests or two sample t-tests for no-choice tests, respectively. Replicates were omitted from analysis if there was no feeding on either treatment. Chi-square tests also were used to compare total proportions of treated or untreated blades with some feeding damage. Statistix 8 (Analytical Software, 2008) was used for all statistical analyses except for weighted ANOVAs, for which SAS (SAS Institute, 2005) was used. Percentage data were normalized by arcsine square-root transformation for analysis. All data are reported as original (non-transformed) means ± SE.

**Results**

**Evaluating Virus/fungicide Combinations in Small Field Plots.** Few larvae were recovered from fungicide-treated plots regardless of whether or not virus was included in the treatment (Table 3). The percentage of recovered larvae that ultimately died from viral infection increased at higher virus rates with a significant linear trend for rate. Lower rates of virus infection occurred in combination treatments than with virus alone resulting in a significant fungicide by virus interaction; however, there was no main effect of fungicide on percent mortality (Table 3).

**Testing for Direct Insecticidal Effects of Fungicide.** Unlike the first experiment, wherein the relatively small number of larvae recovered from fungicide-treated plots had suggested mortality from the fungicide itself, or proportionately more escapes from those enclosures, similar numbers of larvae were recovered from fungicide treated and untreated plots (control = 15.3 ± 0.6; high fungicide rate = 18.0 ± 0.9; low
fungicide rate = 15.7 ± 1.5; \( F_{2, 10} = 2.9; P = 0.1 \). This indicates that the fungicide itself did not have direct adverse effects on larval survival.

**Testing Fungicide/virus Synergism; Greenhouse Trials.** When larvae were exposed to treated turfgrass in the greenhouse for 24 hours the number recovered from the pots was similar for all treatments. Percentage mortality from virus infection increased as virus rate increased, but was similar for the two lowest virus rates. There was no significant main effect of fungicide (Table 4). A virus × fungicide interaction was seen; however, when fungicide/virus combinations were compared to comparable rates of virus alone, the percentage mortality from virus was similar regardless of whether or not fungicide was included.

When larvae were exposed to treated grasses for 1, 2 and 4 days the number recovered from the pots again was similar for all treatments. Longer duration of feeding on treated grasses and exposure to the higher virus rates corresponded to greater mortality from virus infection (\( F_{2, 67} = 4; P = 0.02; F_{2, 67} = 115, P \leq 0.01, \text{ respectively} \)) for all exposure durations (Table 5). Within rates, larvae exposed to the low rate of virus alone experienced significantly higher mortality (41.8 ± 4.7 versus 22.1 ± 5.5; \( F_{1, 24} = 10.1; P \leq 0.01 \)) and died more quickly compared to larvae feeding on grasses treated with the low-virus/fungicide combination for all exposure times (Figure 6; Table 5). Larvae also died more quickly at the high virus rate compared to high-virus/fungicide combinations when exposed for 2 and 4 days; however, rate of death was similar when exposed for only 1 day (Figure 6; Table 5).

**Fungicide Effects on Consumption of Treated Grass.** In choice tests with neonates, the total number of grass blades with some damage caused by cutworm feeding
was similar for the treated and the non-treated grasses (52 versus 58; $\chi^2 = 0.33; P = 0.56$). Larvae consumed proportionately less of the treated than of the non-treated grass tissue (17.1 ± 1.8% versus 24.7 ± 1.7%, respectively; Wilcoxon signed-rank test, $P \leq 0.01$). Numbers of larvae feeding on treated versus untreated grass blades were similar at the time of assessment, however. In no-choice tests with neonates, the percentage feeding damage on treated grass blades (8.5 ± 2.6%) was significantly lower than for non-treated blades (22 ± 3.9%; $t_9 = -2.76; P = 0.01$), but the number of blades with some cutworm damage was similar regardless of treatment. Third instars showed significant preference for non-treated grass blades in choice tests (Fig. 7).

**Discussion**

Combined or overlapping applications of a labeled polyoxin-d fungicide and *Agip*MNPV would be practical in turfgrass settings, so it was hoped that the combination would enhance infectivity of the virus against the black cutworm, an important golf course pest, compared to levels of control provided by virus alone. That hypothesis is reasonable given previous laboratory studies with other insect species in which chitin synthesis-inhibiting agents facilitated passage of virions through the chitinous peritrophic membrane, enhancing viral infection (Becker 1980, Arakawa 2002; 2003, Hui-Fang et al. 2007). However, we saw no synergism by the chitin synthesis-inhibiting fungicide; instead, there was delayed and slightly reduced mortality from *Agip*MNPV when larvae fed on fungicide/virus treated grasses compared to virus-only treatments.

Poor recovery of larvae from fungicide-treated plots in the first field experiment initially suggested that polyoxin-d might have an insecticidal effect on cutworms.
However, in a second experiment when metal enclosures were driven deeper into the turf, similar numbers of larvae were recovered from fungicide and non-fungicide treated plots, revealing that the fungicide does not kill the cutworms. In choice tests, cutworms avoided feeding on polyoxin-d treated grass. This suggests that larvae disproportionately escaped from the fungicide-treated turf by crawling beneath the shallow-driven enclosures used in the first field experiment. Because polyoxin-d does not deactivate AgipMNPV, and high virus rates can knock down and overwhelm cutworm populations in the short term (Bixby-Brosi and Potter 2010), the two substances are compatible and can be used together in the field. However, polyoxin-d residues on treated grass might interfere with larval ingestion of a lethal virus dose by inhibiting feeding or repelling larvae from putting greens, tees, or other treated sites.

Previous studies examining the insecticidal effects of chitin synthesis inhibitors have all been done in laboratory settings and involved direct injection of the compound into the insect or incorporating it into artificial diet (Gijswijt et al. 1979, Nishioka et al. 1979, Cohen and Casida 1982, Gelman and Borkovec 1986, Arakawa et al. 2007). To our knowledge this is the first study to examine the use of a chitin synthesis inhibitor as a synergist to an entomopathogen on living plants in greenhouse or field settings. Adjuvants such as stilbene optical brighteners that have been shown to protect baculoviruses from UV degradation, enhance their longevity, or act as synergists to virus infection in laboratory studies, may or may not provide the same benefits in the field (Ignoffo and Batzer 1971, Shapiro 1992). The optical brightener M2R, for example, reduced the LD$_{50}$ value of AgipMNPV to A. ipsilon in the laboratory but failed to enhance its efficacy against the same pest in greenhouse- or field-grown corn (Zea mays L.)
Optical brighteners also failed to accelerate or extend efficacy of *AgipMNPV* against *A. ipsilon* in turfgrass field plots (Bixby-Brosi and Potter 2010).

Possibly, polyoxin chitin synthesis inhibitors consumed on plant tissue are less disruptive to caterpillars’ peritrophic membranes than when ingested in artificial diet. Plant secondary chemicals can alter susceptibility of insects to naturally encountered pathogens as well as to microbial insecticides applied for biological control (Cory and Hoover 2006). Caterpillar mortality, for example, can differ by as much as 50-fold depending on the species of host plant upon which baculoviruses are consumed (Keating et al. 1988, Hoover et al. 1998, Ali et al. 1998).

We are still optimistic that *AgipMNPV* has potential as a microbial insecticide for managing black cutworms on golf courses, sports fields, and in garden crops. Selecting for more virulent strains, or formulating the virus with adjuvants that enhance its persistence in field settings could be productive. Testing *AgipMNPV* in combination with other chitin synthesis-inhibiting fungicides suited for golf course use is warranted, because some may be more disruptive to peritrophic membranes without discouraging feeding on treated grass as occurred with polyoxin-d. Another approach might be to combine a high dose of virus with a short-lived natural feeding stimulant (Farrar et al. 2005, Lasa et al. 2009) so that targeted larvae more rapidly ingest a lethal dose. The commercial success of *AgipMNPV*, like most entomopathogens, largely depends on future development of in vitro production methodology allowing the virus to be produced more economically and in greater amounts (Black et al. 1997, Kompier et al. 1988).
Table 3. Numbers of *A. ipsilon* recovered from small-plot field experiments, and percentage that died from virus infection

<table>
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<tr>
<th>Rate (OB m$^{-2}$)</th>
<th>Fungicide</th>
<th>Larvae recovered</th>
<th>% mortality$^a$</th>
</tr>
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<tr>
<td>0</td>
<td>–</td>
<td>11.3 ± 2.3</td>
<td>1.5 ± 1.5</td>
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<tr>
<td></td>
<td>+</td>
<td>3.3 ± 1.7</td>
<td>4.8 ± 4.8</td>
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<tr>
<td>$5 \times 10^6$</td>
<td>–</td>
<td>4.1 ± 2.0</td>
<td>8.5 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.5 ± 1.3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>$5 \times 10^7$</td>
<td>–</td>
<td>8 ± 3.2</td>
<td>24.3 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.3 ± 1.9</td>
<td>7.8 ± 5.1</td>
</tr>
<tr>
<td>$5 \times 10^8$</td>
<td>–</td>
<td>6.5 ± 1.9</td>
<td>61.7 ± 14.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.0 ± 1.0</td>
<td>47.7 ± 21.4</td>
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ANOVA (*F* values)$^b$

<p>| | | | |</p>
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<tbody>
<tr>
<td>Fungicide</td>
<td>11.7**</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Virus rate</td>
<td>1.6</td>
<td>18.4**</td>
<td></td>
</tr>
<tr>
<td>$F \times V$</td>
<td>0.8</td>
<td>6.1*</td>
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</table>

$^a$ Weighted ANOVA

$^b$ df: 1, 3, 3, 35 for fungicide, virus rate, interaction, and error, respectively

* and ** denote significance at $P \leq 0.05$ and 0.01, respectively.
Table 4. Numbers of larvae recovered from virus/fungicide treated pots after 24 hours of feeding, and percentage infected by virus in greenhouse trials.

<table>
<thead>
<tr>
<th>Rate (OBs)</th>
<th>Fungicide</th>
<th>Larvae recovered</th>
<th>% mortality&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>High</td>
<td>10.3 ± 0.8</td>
<td>1.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>9.8 ± 0.9</td>
<td>4.8 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>10.0 ± 0.4</td>
<td>4.8 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>11.2 ± 0.3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>5×10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>High</td>
<td>10.7 ± 1.0</td>
<td>17.1 ± 4.7</td>
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<tr>
<td></td>
<td>Medium</td>
<td>8.8 ± 0.5</td>
<td>1.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>7.1 ± 0.8</td>
<td>17.5 ± 9.2</td>
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ANOVA (<i>F</i> values)<sup>b</sup>

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<sup>a</sup>ANOVA

<sup>b</sup>df: 3,3,9,75 for fungicide, virus rate, interaction, and error, respectively

* and ** denote significance at <i>P</i> ≤ 0.05 and 0.01, respectively.
Table 5. Analysis of variance for main effects and interaction of virus rate, fungicide, and days of exposure on percentage of black cutworms that died from viral infection.

<table>
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<tr>
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<td>2.7</td>
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<tr>
<td>4 days&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.3**</td>
<td>1.6</td>
<td>19.17**</td>
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<td></td>
<td>Fungicide</td>
<td>Virus × Time</td>
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<td>0.1</td>
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</table>

<sup>a</sup> df = 2, 1, 10, 5, 120 for virus rate, fungicide, interactions, and error, respectively

<sup>b</sup> df = 2, 1, 2, 8, 4, 96 for virus rate, fungicide, interactions, and error, respectively

<sup>c</sup> Preplanned single degree of freedom contrasts

* and ** denote significance at P ≤ 0.05 and 0.01, respectively.
Figure 6. Cumulative lethal virus infection for *A. ipsilon* fed on bentgrass cores treated with two rates of *Agip*MNPV (VL = Low-virus, $1 \times 10^8$ and VH = High-virus, $5 \times 10^8$ OB/m$^2$) and one fungicide rate (F = 2.1 g m$^{-2}$ of formulated product), applied alone and in combination. Larvae were exposed to treated grasses for 1, 2, and 4 days. Data are means (± SE). Delayed and slightly reduced mortality from *Agip*MNPV occurred when larvae fed on fungicide/virus treated grasses compared to virus-only treatments for all cases, except those in which larvae were exposed to the high virus rate for 1 day.
Figure 7. Mean (± SE) percentage of fungicide-treated versus non-treated grass leaf tissue consumed by third instar *A. ipsilon* in choice tests. Asterisks denote significant feeding preference for untreated grass (Wilcoxon signed-rank tests, *P* < 0.05). The trend after 1 hour was significant at *P* = 0.08.
Chapter Four

Endophyte-Mediated Tritrophic Effects on Two Parasitoids Having Different Life History Strategies That Exploit a Grass-Feeding Caterpillar

Introduction

Tritrophic interactions among plant secondary chemicals, insect herbivores, and parasitoids have been extensively explored (Price et al. 1980, Barbosa and Saunders 1985, Turlings and Benrey 1998, Heil 2008). Constitutive and induced phytochemicals can serve as kairomones that facilitate parasitoid foraging behavior, host finding, and acceptance (Nordlund et al. 1988, Turlings et al. 1992, De Moraes et al. 1998, Turlings and Benrey 1998, Fukushima et al. 2002, Heil 2008). Parasitoid fitness parameters such as survival to eclosion, adult weight, longevity, and clutch size may be compromised when host body size is stunted as a result of phytochemicals (Meijden 1980, Slansky 1986) or when larval parasitoids encounter ingested plant toxins in the bodies of their hosts (Campbell and Duffey 1979, Duffy et al. 1986, Price 1986, Thorpe and Barbosa 1986, Barbosa et al. 1991, Gauld and Gaston 1994, Lampert and Bowers 2010). Only a few studies, however, have compared the effects of plant resistance factors on parasitoids that attack the same host, yet have different life histories (Farrar et al. 1992, Farrar and Kennedy 1993, Kennedy 2003).

Koinobiont endoparasitoids spend most or all of their larval development inside a single actively feeding host insect, only to consume or kill it once the parasitoid reaches a certain stage (Mackauer and Sequeira 1993, Godfray 1994). With some, only the larval
stage develops in direct contact with a host, whereas others synchronize their development from egg to adult within a host (Mackauer and Sequiera 1993, Godfray 1994). Solitary parasitoids often kill hosts earlier in larval development than gregarious ones, whose offspring need more resources for development (Senthamizh selvan and Muthukrishnan 1989). Plant resistance and biological control by parasitoids are important tactics in many integrated pest management systems so it is important to understand how parasitoids having different life history strategies are affected by toxins or other secondary chemicals ingested or sequestered by their plant-feeding hosts.

Several grasses (Poaceae) form symbiotic relationships with endophytic fungi, *Neotyphodium* spp., which grow intercellularly within their leaf and stem tissues (Siegel et al. 1987, Schardl et al. 2004). The endophyte derives nutrients from its host plant while producing alkaloids that enhance resistance of the grass to herbivory (Breen 1994, Siegel and Bush 1996, Clay 1997, Schardl et al. 2004). *Neotyphodium lolii*, the endophyte associated with perennial ryegrass (*Lolium perenne* L.), produces three classes of alkaloids: ergot alkaloids, peramine, and lolitrems, contributing in varying degrees to protect from herbivores (Siegel and Bush 1996, Bush et al. 1997, Schardl et al. 2006, Potter et al. 2008). Because endophyte-infected grasses are deterrent or toxic to non-adapted herbivores, including certain grass-feeding insects (Siegel et al. 1987, Clay 1991, Breen 1994), establishing or overseeding endophyte-infected grasses on lawns, sport fields, or golf courses can be useful in suppressing certain leaf- and stem-feeding pests (e.g., Breen 1994, Richmond et al. 2000). However, herbivores such as the black cutworm, *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae), are endophyte-adapted and can feed and complete their development on endophytic grass. Such larvae develop more
slowly than those that feed only on endophyte-free grass (Williamson and Potter 1997, Kunkel and Grewal 2003, Potter et al. 2008).

Although less well-studied than effects on herbivores, the alkaloids produced by fungal endophytes can also affect natural enemies. For parasitoids, such multitrophic effects are mediated by both sequestration and transmission of mycotoxins through food webs and by small size and poor nutritional quality of hosts feeding on endophytic grasses. Effects range from reduced growth, survival, or fecundity of individual species (e.g., Barker and Addison 1996; 1997, Bultman et al. 1997) to altered energy transfer from plants to higher trophic levels affecting whole parasitoid communities (Omacini et al. 2001).

This study examines the intensity of endophyte-mediated tritrophic effects on two parasitoid species having different life history strategies that exploit a common host, *A. ipsilon*, in turfgrass settings (Bixby-Brosi and Potter 2010). The fly, *Linnaemya comta* (Fallen) (Diptera: Tachinidae) (formally: *Bonnetia comta* [Fallen]; O’Hara and Wood 2004), is a rapidly developing solitary parasitoid wherein only the larval stage develops within the caterpillar host. The polyembryonic wasp, *Copidosoma bakeri* (Howard) (Hymenoptera: Encyrtidae) develops more slowly from egg to adult within its host, producing large broods of clonally identical offspring (Saeki et al. 2009). We predicted that both parasitoid species would be adversely affected when developing within a cutworm that feeds on endophytic grass, but that the consequences would be greater for *C. bakeri* because of its relatively longer and more intimate relationship with the caterpillar host.
Materials and Methods

**Cutworms and host plants.** *Agrotis ipsilon* eggs and larvae were obtained from a commercial insectary (Benson, Carlisle, PA, USA) where they had been maintained on a soybean-based diet. Larvae were shipped in cups of diet by overnight mail and transferred to our assays within a few hours of arrival. Eggs were shipped on white cotton fabric and typically hatched within three days of arrival. All experiments were conducted in environmental chambers at 27 ± 0.5 °C (daytime), 25 ± 0.5 °C (nighttime), and 14:10 (L:D) hour photoperiod.

‘Rosalin’ perennial ryegrass, either infected by *N. lolii* or endophyte-free (hereafter referred to as E+ or E−, respectively) was planted in 11.4 × 11.4 cm plastic pots (3 mg seed cm⁻²) in a glasshouse. Seed was obtained from C. L. Schardl (Department of Plant Pathology, University of Kentucky). Endophyte infection, determined by staining representative tillers with an aniline blue solution (Shelby and Dalrymple 1987) was 88% and 0% for E+ or E− grass, respectively. Alkaloid concentrations in the potted E+ grass were not determined in this study; however, tillers grown from the same batch of seeds were previously reported (Potter et al. 2008) to have the following amounts (μg g⁻¹ dry weight) in their grass blades: ergovaline (1.24 ± 0.12), ergine (0.12 ± 0.02), chanoclavine (1.43 ± 0.19), 6,7-secolysergine (0.77 ± 0.08) and peramine (135 ± 58).

Grasses were maintained under 14-hour photoperiod with supplemental lighting from 1000 W sodium vapor bulbs unless ambient light was ≥450 μmol m⁻² s⁻¹. Day and night temperatures were set at 22 and 18 °C, respectively. The potting mix consisted of 3:1 Pro-Mix BX (Premier Horticulture, Quakertown, PA) and autoclaved topsoil. Plants
were fertilized with Peters 20-10-20 Peat-Lite formula (Scotts, Marysville, OH), trimmed to 5 cm, and watered as needed.

**Parasitoid biology.** *Linnaemya comta* is a solitary larval parasitoid generally associated with lepidopteran caterpillars (particularly members of the family Noctuidae), including *A. ipsilon* (Crumb 1956, Arnaud 1978). The female flies, attracted by kairomones in host frass, deposit mobile first-instar larvae (planidia) at the entrance of black cutworm burrows where the larvae wait for a host to come out to feed (Rubink and Clement 1982). Planidia enter the host by using their sharp labrum to cut through its integument, and once inside, attach to the host’s tracheal system to feed. Third instar larvae leave the largely consumed cutworm cadaver to pupate in the soil (O’Hara 2008, Wood 1987).

*Copidosoma bakeri* is a polyembryonic egg-larval parasitoid that oviposits into the eggs of its lepidopteran hosts, which include at least 19 noctuid species (Schaaf 1972, Byers et al. 1993). Development of a polyembryonic egg to produce hundreds to thousands of clonal individuals is synchronized with host development (Strand 1989a). After *C. bakeri* parasitizes a host egg, the host hatches and begins developing normally, while the wasp egg divides multiple times (Ivanova-Kasas 1972). When the host reaches the penultimate larval stadium, the wasp larvae start morphogenesis and growth (Baehrecke and Strand 1990). During the host’s final larval stadium, the wasp larvae devour the host and pupate (Strand 1989b). When wasps form cocoons, the host is mummified and dies (Strand 1989a).
**Parasitoid colonies.** *Linnaemya comta* parasitizing *A. ipsilon* were collected at the University of Kentucky Turfgrass Research Facility (Fayette County, KY, USA) from July to September 2009, and maintained for three generations before the start of the experiments. This was done by confining sentinel 3rd instar cutworms in circular metal enclosures (39.0 cm in diameter by 10.2 cm in height) driven 2 cm into the ground in a stand of creeping bentgrass (*Agrostis stolonifera* L.). The cutworms were recovered from the field after 1 week by using a soap flush consisting of 1.3 ml of lemon-scented dishwashing detergent (Joy® Proctor & Gamble, Cincinnati, OH) per liter of water (Potter 1998). Larvae were rinsed with water as soon as they surfaced and placed in individual capped 30-ml cups with soybean-based noctuid diet (Blanco et al. 2009). Larvae were maintained in an environmental chamber (27 ± 0.5, 25 ± 0.5 °C; 14:10 (L:D) hour photoperiod) until adult *L. comta* emerged.

*Linnaemya comta* fly colonies were maintained in round cages (20 cm diameter, 20 cm height) on 10% honey-water solution (Levine and Clement 1981). Mated females were induced to deposit planidia by exposing them to fresh host frass in open petri dishes (90 ×15 mm) lined with filter paper. Frass was collected from *A. ipsilon* feeding on E—perennial ryegrass clippings. Parasitism was accomplished by transferring individual fly larvae from frass plates to the dorsal surface of healthy third instar cutworms using forceps. The parasitoid larva was observed until it penetrated the host cuticle, to confirm that parasitism occurred. Parasitized cutworms used to maintain the *L. comta* colony were maintained in individual capped 30-ml cups with artificial diet.

*Copidosoma bakeri* colonies were established from *A. ipsilon* eggs parasitized on 20 July 2010 at the University of Georgia, M. R. Strand lab. Eggs were sent via
overnight mail in petri dishes, hatched in an environmental chamber, and reared individually in capped 30-ml cups with artificial diet. Parasitized cutworms that progressed to the mummy stage (less than 5%) were placed in glass centrifuge tubes (1.5 cm diameter × 14.5 cm) and capped with a cotton stopper until adult wasps emerged. Using an aspirator, newly emerged and mated females were transferred to closed petri dishes (50 × 10 mm) containing A. ipsilon eggs. Wasps were left in dishes for 24 hours to allow for adequate oviposition. Newly hatched cutworms were reared as described above. Colonies were maintained for one generation before the start of experiments.

**Endophyte effects on L. comta.** Agrotis ipsilon larvae were raised on artificial diet until they reached third instar, at which point they were weighed, blocked by weight, and placed into individual 30 ml cups containing either E+ or E− grass clippings. After 24 hours of feeding, each larva was removed from grass, placed in empty cups, and one planidial fly larva was placed onto its dorsal surface. Parasitism was validated by viewing through a binocular stereomicroscope. Parasitized and unparasitized cutworms (five replicates of 20 individuals per group) were then fed either E+ or E− grasses for the duration of their larval development. Larvae were weighed 7 and 10 days after parasitism. Fly pupae and adults were weighed and the number of days until flies pupated and eclosed was recorded. The sex ratio of emerging flies was also determined. As an indicator of host feeding activity, all frass produced by feeding larvae between days 10 and 12 was collected, air dried, and weighed.

**Endophyte effects on C. bakeri.** Mated female wasps were transferred to closed petri dishes (5 × 1 cm), containing 10–20 black cutworm eggs. Wasps were monitored through a binocular stereomicroscope to observe oviposition activity. Eggs that appeared
to be parasitized were marked and all others were discarded. This process was repeated until at least 120 eggs had been parasitized. Parasitized and unparasitized eggs were hatched, and neonates were placed into multiple containers (11 cm diameter × 14 cm) with E− grass or E+ grass. After 10 days of feeding, cutworm larvae were removed from the containers, weighed, and then placed individually into 30 ml cups containing either E− or E+ grass (30 cutworms per grass type). Larvae were weighed every 2–4 days. For cutworms that died as larvae or developed normally, the number of days until death or pupation was recorded. Percentage of successful parasitism, mummy weights of parasitized hosts, and numbers, average weight, and sex of emerged wasps in each brood were recorded.

Parasitized hosts were fed either E+ or E− grass throughout the entire duration of parasitoid development, for both *C. bakeri* and *L. comta*. However, because these parasitoids have different life history strategies, *C. bakeri* hosts were fed E+ or E− grass from egg hatch, whereas *L. comta* hosts started feeding on grasses as 3rd instar larvae. Because *A. ipsilon* larvae fed E+ grasses are developmentally stunted, and to avoid confounding our results by starting with hosts of two different sizes, *L. comta* hosts were fed artificial diet until 24 hours before parasitism.

**Statistical analysis.** Cutworm larval and frass weights were analyzed using a 2 × 2 factorial analysis of variance (ANOVA) for main effects and interaction of endophyte and parasitism. One-way ANOVA was conducted to determine endophyte effects on cutworm and parasitoid pupae and adult weights. A two-way ANOVA for repeated measures was used on cumulative percentages of *L. comta* that pupated and eclosed. Fixed factors were endophyte (between-subjects factor) and time after parasitism (within-
subjects factor), with percentage that had pupated or eclosed as the repeated measure over time. Percentages were recorded on groups of flies within the same replicates over time. Percentage data were normalized by arcsine square-root transformation before analysis. Chi-square tests were used to compare total proportions of male and female *L. comta* eclosed from cutworms that had consumed either E+ or E− grass. Statistix 8 (Analytical Software 2008) was used for all statistical analyses. All data are reported as non-transformed values, either as means ± standard error (SE) or actual counts.

**Results**

**Endophyte effects on *L. comta***. Cutworms reared on E+ grass weighed significantly less than their counterparts that fed on E− grass after 10 days; however, weights were similar in parasitized and unparasitized groups (Table 6). Percentage successful parasitism was similar for both the E− and E+ cohorts (65 ± 1.6 versus 73 ± 4.6%, respectively; *F* = 2.4; df = 1, 4; *P* = 0.2). Development of the parasitoid was similar regardless of the type of grass the host consumed; i.e., endophyte did not significantly affect the number of days required for the flies to pupate (*F* = 0.6; df = 1, 48; *P* = 0.45) or to emerge as adults (*F* = 0.9; df = 1, 32; *P* = 0.4; Figure 8). On average, flies pupated at 12 ± 0.1 days and eclosed at 25.5 ± 1 day after parasitism. The weight of fly pupae and adults was similar regardless of grass type (Table 6; Figure 8).

Additionally, sex ratios were similar for flies that emerged from hosts that had consumed E− or E+ grass (29:37 [F:M] versus 21:35, respectively; *χ*² = 0.5; df = 1; *P* = 0.5).

Parasitized hosts produced more frass than unparasitized cutworms between days 10 and 12 of the experiment (40 ± 2 versus 27 ± 2 mg respectively; *F* = 16.2; df = 1, 92; *P* ≤ 0.01); however, frass weight was similar regardless of whether the caterpillars were
feeding on E− or E+ grass (3.1 ± 0.2, 3.6 ± 0.2, respectively; \( F = 2.3, \ df = 1, 94, P = 0.09 \)).

**Endophyte effects on C. bakeri.** Ten days after egg hatch, larvae feeding on E+ grass again had significantly lower weights than E− fed cutworms (7.6 ± 0.4 versus 17.5 ± 1.7 mg respectively; \( F = 29.1; \ df = 1, 112; P \leq 0.01 \)), and that difference was accentuated over time. Unparasitized larvae feeding on E− grass experienced higher pupation success than E+ fed larvae (Figure 9).

Parasitism of both E− and E+ fed larvae was less than expected; i.e., not all of the eggs visited by female C. bakeri and presumed to be parasitized based on the wasps’ oviposition behavior gave rise to hosts that supported internal development of a parasitoid brood once the study was underway. The number of presumed parasitized cutworms (initially 30 per cohort) in which a wasp brood developed was lower for hosts that fed on E+ as opposed to E− grass (3 versus 11, respectively, \( \chi^2 = 4.5; \ df = 1; P = 0.03 \), Figure 10). Of those E+ fed cutworms in which a parasitoid brood developed, one died and the other two continued to develop, abnormally, to the prepupal stage before mummies were formed. Those mummies consequently were much smaller than the mummies (\( n = 11 \)) of parasitized hosts that had fed on E− grass (226.5 ± 7.5 versus 345.8 ± 22.2 mg, respectively). On average, E− mummies formed more quickly than E+ mummies (31 ± 1 versus 49.5 ± 1.5 days after egg hatch, Figure 10) and contained more C. bakeri adults per brood (748 ± 119 versus 121). All broods that emerged from E− mummies were unisexual, (1 female brood and 10 male broods), whereas the only brood that emerged from an E+ mummy was mixed-sex. Mixed-sex broods occur when two eggs are deposited into a host egg, developing simultaneously (Ode and Strand 1995).
Discussion

Tritrophic interactions involving parasitoids and caterpillars can take a variety of forms depending on the biological attributes of the insects’ relationship and the type of plant upon which it occurs (Kennedy 2003). The results of this study support our prediction that *C. bakeri*, a polyembryonic wasp that has a relatively long and intimate relationship with the host, would suffer greater negative fitness effects than would *L. comta*, a solitary, rapidly-developing tachinid fly, when their common host feeds on alkaloid-containing endophytic grass. Indeed, proportionately fewer parasitized cutworms yielded *C. bakeri* broods when the caterpillars consumed E+ grass, those host mummies were smaller and slower to form, and they contained fewer *C. bakeri* adults per brood than those broods from hosts developing on E− grass. The tachinid, in contrast, did not appear to be affected by the presence of endophyte infection within its hosts’ plant.

The aforementioned differences may reflect the different manners in which the two parasitoid species interact with their host. *Linnaemya comta* parasitizes the third or fourth instars, entering the host as a planidial first instar, completing larval development, and emerging to pupate before host pupation, whereas *C. bakeri* inserts its egg into a host egg, develops slowly, and spends a greater amount of time in direct contact with its host. Other *Copidosoma* species also have shown negative fitness effects in response to phytochemistry, reflecting either reduced host size and quality or direct exposure to phytotoxins in host hemolymph (Orr and Boethel 1985, Beach and Todd 1986, Reitz and Trumble 1996, Lampert et al. 2011). In our study, cutworms were developmentally
stunted when feeding on E+ grass, making it difficult to separate host size effects from those resulting from direct exposure of the parasitoids to ingested alkaloids in the host’s gut or hemolymph.

Our finding that *L. comta*-parasitized hosts produce more frass, regardless of whether they feed on E+ or E− grass, is suggestive of compensatory feeding. The tachinid’s ability to develop normally in hosts feeding on E+ grass may reflect tolerance to associated alkaloids, or simply that even a stressed host adequately supports the fly’s relatively rapid development. A study evaluating effects of baculovirus infection of *A. ipsilon* on *L. comta* development showed no detrimental effects on the parasitoid except when the virus killed the cutworm before the fly maggot was able to emerge from the host cadaver (Cossentine and Lewis 1986). *Linnaemya comta* maggots from infected cutworms contained viral occlusion bodies in their gut lumens, yet their development was similar to those in non-infected hosts (Cossentine and Lewis 1986). Because the tachinid’s planidial larvae attack third or fourth instar hosts, and develop so quickly, host size may be less critical than it is for successful brood development of *C. bakeri*.

The two parasitoid species discussed are natural enemies of *A. ipsilon* in turfgrass settings where their host may encounter and feed on E+ perennial ryegrass, as well as tall fescue, *Festuca arundinacea* Shreb., infected with its alkaloid-producing endophyte, *Neotyphodium coenophialum*. Use of endophytic turfgrasses has been suggested for sustainable management of certain endophyte-sensitive insect pests (Breen 1994, Richmond et al. 2000), so it is desirable that it not disrupt the endemic natural enemies of endophyte-tolerant species such as *A. ipsilon* that occur at the same sites. A study combining biological control of *A. ipsilon* by a baculovirus and endophytic grass
resistance determined that the two pest management strategies are compatible (Bixby and Potter 2010). However, delayed and slightly reduced mortality from the virus was observed when larvae fed on virus-sprayed E+ as opposed to E− ryegrass. That study suggests that reduced consumption or avoidance of virus-contaminated E+ grass could affect the likelihood that *A. ipsilon* will encounter or ingest a lethal dose of virus in the field (Bixby and Potter 2010). Our study suggests that endophytic grass resistance may be more compatible with some types of parasitoids than others, but more research is needed to determine if hymenopteran parasitoids, or those having a polyembryonic or gregarious life history are, as a group, more endophyte-sensitive than are dipteran or solitary parasitoid species.

Endophyte effects on *C. bakeri* also warrant further study. A colony collapse occurred when only all-male broods emerged from the *A. ipsilon* mummies, preventing us from repeating and expanding upon the *C. bakeri* experiment to clarify at what stage in the parasitoid’s complicated life history within the host its development was disrupted. Regardless of that shortcoming, this study highlights that tritrophic interactions between caterpillars, parasitoids, and plant secondary chemistry can be strongly influenced by the life history strategies or the taxonomic group of the parasitoids involved.
Table 6. Weights (mg) of unparasitized versus *L. comta*-parasitized black cutworms after feeding for 10 days on non-endophytic (-) or endophytic (+) perennial ryegrass, and of the resulting parasitoid pupae, and adult flies.

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<th>Fly pupae$^b$</th>
<th>Adult flies$^c$</th>
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<td>-</td>
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<tr>
<td>+</td>
<td>-</td>
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ANOVA (*F* values)

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</tbody>
</table>

$^a$Cutworms weighed 10 days after the parasitized group had been penetrated by planidial larvae of *L. comta*; n = 100; df = 1, 1, 1, 384 for endophyte, fungicide, interaction, and error, respectively

$^b$n = 67, 73; df = 1, 130 for endophyte and error respectively

$^c$n = 58, 67; df = 1, 118 for endophyte and error respectively

*and** denote significant at $P \leq 0.05$ and 0.01, respectively.
Figure 8. Weights of *A. ipsilon* larvae and pupae, and *L. comta* pupae and adults emerged from parasitized (P) hosts fed endophytic (E+) and endophyte free (E−) grass over time. Parasitoid weights after exiting the host are delineated by the dotted box. Labels on the X-axis refer to days after parasitism.
Figure 9. Weights of unparasitized *A. ipsilon* and counterparts parasitized by *C. bakeri* (P). Both groups were reared on either endophytic (E−) and endophyte free (E+) grass. The X-axis shows days after *A. ipsilon* egg hatch. Unparasitized larvae feeding on E− grass developed normally and pupated about 20 days after egg hatch. Host mummies containing *C. bakeri* were formed about 27 days after egg hatch of E− fed hosts. Image on left depicts *A. ipsilon* pupa, image on right depicts a mummy. Only weights from parasitized larvae were included; E−P (n = 11), E+P (n = 3). Hosts fed E+ grass remained larvae throughout the time period shown.
Figure 10. Numbers of parasitized (P) or unparasitized *A. ipsilon* that either pupated, died as larvae, or gave rise to a *C. bakeri* brood when the hosts were reared on endophytic (E+) or endophyte free (E−) grass (n = 30 for each treatment).
**Conclusion**

Turfgrasses are the most intensively managed plantings in urban landscapes, and high aesthetic standards result in substantial use of pesticides on home lawns, sports fields, and golf courses. Traditional management of turfgrass insect pests has relied mostly on the use of chemical insecticides that have the potential to adversely affect beneficial organisms. Increased restrictions on the use of pesticides in urban and suburban areas, loss of insecticide registrations, increased public concerns about pesticides, and industry wide initiatives in environmental stewardship leave turfgrass managers in need of alternative means of control. Present options for microbial insecticides and inoculated release of natural enemies are limited in turfgrass pest management, but the potential for turfgrass managers to practice conservation biological control is high.

Hurdles to the use and commercialization of microbial turfgrass insecticides include their narrow spectrum of activity, relatively high cost, short shelf life, problems with in vitro production, and competition from highly effective synthetic insecticides. *Paenibacillus popilliae* (Dutky), causal agent of milky disease in Japanese beetle (*Popillia japonica* Newman) grubs, and entomopathogenic nematodes presently are marketed in the United States as microbial insecticides. *Bacillus thuringiensis* Berliner products are labeled against grass-feeding caterpillars but are rarely used by the turf industry because of their short residual and poor activity against larger larvae. A product with the fungus *Beauveria bassiana* (Balsamo) was briefly marketed in the 1990s but withdrawn due to inconsistent performance.
Baculoviruses could realistically compete with chemical insecticides for managing *A. ipsilon* or other grass-feeding caterpillars on golf courses or sport fields if one application provided extended suppression once the virus became established in the turfgrass. In this study, fresh AgipMNPV residues killed a high percentage of *A. ipsilon* in all heights of turf, but efficacy lasted only a few weeks. When AgipMNPV was combined with adjuvants, such as optical brightener and lignin, I did not see an acceleration or extension of efficacy of the virus. Realistically, golf courses are a severe environment for entomopathogens. Daily or frequent mowing and clipping removal, irrigation, and other intensive management practices are not conducive to maintaining lethal titers of baculoviruses on grass foliage. AgipMNPV could, however, be used to knock down existing cutworm populations, especially if the time of application coincided with early instars. Higher virus concentrations and multiple applications per growing season may be required to manage cutworms or other grass-feeding caterpillars on such high-profile sites as putting greens.

My work with AgipMNPV plus the work done by Prater et al. (2006) is the first to investigate the use of a baculovirus in a turfgrass setting. For our trials we were limited to spraying a self-made crude AgipMNPV suspension. Commercial success of AgipMNPV would be facilitated by advances in in vitro production or other methods allowing the virus to be produced more economically and in greater amounts. Despite the aforementioned hurdles, turf provides a strong potential market for biological insecticides, and efforts to develop AgipMNPV for sustainable golf course management are warranted. In fact, Sylvar Technologies Inc. (Fredericton, New Brunswick, Canada) and Andermatt Biological Control (Grossdietwil, Switzerland), two companies
specializing in the advancement of baculovirus technologies, are interested in further research and commercialization of AgipMNPV and independently contacted our lab about collaborative development. The intent of these companies has largely been the use of baculovirus in forest and agricultural systems.

The possibility that a substance already being used on golf courses could synergize or extend virus activity was explored. I tried to enhance virus activity by applying it in combination with a chitin synthesis inhibiting fungicide, polyoxin-d, but obtained delayed and slightly reduced mortality from AgipMNPV when larvae fed on fungicide/virus treated grasses compared to virus-only treatments. I also fed A. ipsilon larvae endophytic grass in combination with AgipMNPV to determine compatibility and possible virus synergism. Although the virus was not synergized by the fungicide or the endophytic alkaloids, it was however, not deactivated.

Polyoxin-d and endophytic grasses are compatible with biological control by AgipMNPV, however, larvae preferred to feed on non-fungicide treated grass or endophyte free grass. It is possible that reduced consumption or avoidance of virus-contaminated E+ or fungicide treated grass could affect the likelihood that A. ipsilon will encounter or ingest a lethal dose of virus in the field. Reduced feeding on or migration of larvae from E+ or fungicide treated grasses could somewhat reduce the impact of a virus in field settings. Alternatively, use of E+ tall fescue or perennial ryegrass in surrounds might have net positive results for pest management if the slower development and smaller size of A. ipsilon feeding on such grasses exposed them to greater mortality from predators and parasitoids, or cause them to be a smaller size or at an earlier instar at the time the virus was applied. Endophytes affect both the physiology and behavior of grass-
feeding insects, so the outcome of using combinations of E+ grasses and baculoviruses for pest management may be difficult to predict.

Fungal endophytes may also affect the natural enemies of insect herbivores feeding on virus infected grasses. Little was known about the identity of black cutworm parasitoids in turfgrass habitats or how much they contribute to biological control. In my field surveys nearly 30% of the cutworms collected from golf courses and other turf sites in Kentucky were parasitized. At least five different parasitoid species were found. I examined how feeding on perennial ryegrass with or without its alkaloid-producing fungal endophyte, affects suitability of a noctuid caterpillar, Agrotis ipsilon, as a host for two parasitoid species with different life history strategies. My results suggest that endophytic grass resistance may be more compatible with some types of parasitoids than others, but more research is needed to determine how these interactions will affect parasitoid populations. Clearly these parasitoids, and likely others, contribute to biological control of A. ipsilon in turfgrass, so conservation of their benefits is another reason why a selective, baculovirus-based insecticide would be useful for managing cutworms on golf courses or sport fields. The combination of the baculovirus, parasitoids, and predators has the potential to suppress cutworm populations enough that insecticide usage could be significantly reduced. I hope that by providing groundwork for augmentative and conservation biological control, my work will help the turf industry’s initiatives toward greater environmental stewardship.

Finally, the adoption of biological control methods by the general public has historically been low due to high aesthetic standards and low tolerance for pests. However, public perspective of “aesthetically pleasing” is progressively changing in the
turfgrass industry, resulting in higher demand for non-chemical control options. Potential markets for *Agip*MNPV or other biological insecticides include organic lawn control, sustainably or organically managed golf courses, courses participating in environmental stewardship programs, e.g., as wildlife sanctuaries, sites where surface runoff of chemicals into ponds or streams is a concern, and sport fields or other sites where the use of chemical insecticides is prohibited or poses undue liability. The turfgrass industry consists of many diverse groups including millions of homeowners, athletic field managers, lawn care operators, golf course superintendents, and parks and grounds superintendents, each with a different educational background and tolerance threshold for insect damage. I believe that outreach and extension programs focusing on reducing chemical inputs and making end users aware of the benefits of conserving natural enemies will pave the way to increased use of biological control and public acceptance of its limitations.
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EDUCATION:
M.S., Entomology, University of Rhode Island, Completed: May 2007,
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POSITIONS HELD:

- **Graduate Research Assistant**, Turf and Landscape Entomology Lab, University of Kentucky, Department of Entomology, 2007–2011.

- **Graduate Research Assistant**, Lab of Turfgrass Entomology, University of Rhode Island, Department of Plant Sciences, 2004–2007.


SCHOLASTIC AND PROFESSIONAL HONORS:

- Invited Book Chapter: Beneficial and Innocuous Invertebrates in Turfgrass”
  In Handbook of Turfgrass Insects. 2nd edition. Entomology Society of America, Lanham, MD, in press.
- University of KY, Women’s Club Endowed Fellowship, 2010
- Tracy Farmer Institute for Sustainability and the Environment, University of KY; Karri Casner Environmental Sciences Fellowship, 2010
- University of Kentucky, Department of Entomology Publication Scholarship, 2009, 2010
- Co-organized Symposium: “Building Sustainable Urban Landscapes.”
- University of Kentucky, Graduate School Professional Meeting Travel Scholarship 2009
- University of Kentucky H. Garman Entomology Club: Student Liaison to the Faculty, 2009-2010; President 2008-2009; Social Event Co-chair, 2007-2008.
- Paratech Company-USDA Small Business Grant. 2008. Project Title: Eastern Tent Caterpillar as a biologically based virus production system for

- University of Rhode Island Coastal Fellowship 2000–2001
- Soil Science Society of America, Rhode Island Branch Scholarship, 2000

**PUBLICATIONS:**

**Book Chapter**


**Refereed**


Non-refereed


RESEARCH PRESENTATIONS:

Invited Presentations


Bixby, A. J. & D. A. Potter. Prospects for Managing Turfgrass Insect Pests with Baculoviruses, 2010 International Meeting of the Society for Invertebrate Pathology, Trabzon, Turkey


Bixby, A. J. and D. A. Potter. Use of a Baculovirus for Season-long control of BCW on Golf Courses, 2008, ESA, Reno, NV
**Offered Oral Presentations**

Bixby-Brosi, A. J. and D. A. Potter, Biological Control of a Grass Feeding Caterpillar and Endophyte Mediated Tritrophic Interactions in Turfgrass. 2010, University of Kentucky PhD Exit Seminar. Lexington, KY

Bixby, A. J. and D. A. Potter. Influence of Endophyte (*Neotyphodium lolii*) Infection of Perennial Ryegrass on Susceptibility of the Black Cutworm to a Baculovirus. 2010, North Central Branch Meeting of the Entomological Society of America. Louisville, KY

Bixby, A. J. and D. A. Potter. Tritrophic interactions of a baculovirus and a grass endophyte associated with biological control of the black cutworm (*Agrotis ipsilon*). 2009, Annual Meeting of the Entomological Society of America (ESA), Indianapolis, IN.


Bixby, A. J. and S. R. Alm. Susceptibility of Four Species of Turfgrass-infesting Scarabs to *Bacillus thuringiensis* Serovar *japonensis* Strain Buibui. 2007, National Turfgrass Entomology Workshop, Wooster, Ohio; ESA, San Diego, CA.


**Extension Presentations**


Bixby, A. J. Know Natural Enemies in Turfgrass. Turfgrass Field Day, University of KY Spindletop Research Farm, Lexington, KY 2010


Bixby, A. J. Kentucky Turfgrass Entomology Research Update. Turfgrass Field Day, University of KY Spindletop Research Farm, Lexington, KY 2008 and 2009


Bixby, A. J. and D. A. Potter. Microbial Control of Black Cutworm in Turfgrass using a Naturally Occurring Virus, Kentucky Turfgrass Council meeting, Bowling Green, KY, October, 2007

TEACHING

Agroecology (Plant & Soil Science 597), University of Kentucky

   Guest Lecturer, February 2010 & 2011, enrollment= 12-15

Taught portion of laboratory on biological control, highlighting microbial insecticides in Agroecosystems and my PhD research.

Introduction to Agriculture (Agriculture 100), University of Kentucky

   Guest Lecturer, September 2009, enrollment= 20

   Taught 50 minute lecture on the evolution and history of pesticides agriculture.

Principles of Turfgrass Management (Plant & Soil Science 515), University of Kentucky, Guest Lecturer, September 2008, enrollment=8

   Taught a portion of laboratory focusing on research in turfgrass science, while highlighting the possibilities for microbial insecticides in turfgrass systems.

Horticultural Entomology (ENT 320), University of Kentucky

   Teaching Assistant/Lab Instructor, August-December 2008, enrollment=30.

   Prepared and gave lab introduction and instructions, graded written portions of exams.

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PROFESSIONAL MEMBERSHIP

1) Entomology Society of America, member since 2004.

2) International Organization of Biological Control of Noxious Animals and Plants, Nearctic Regional Section, member since 2008.

3) Society of Insect Pathology, member since 2008.