

2008

# Bt-resistant European corn borers and *Nosema pyrausta*: implications for resistance management

Miriam Dorothy Lopez  
*Iowa State University*

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>



Part of the [Entomology Commons](#)

---

## Recommended Citation

Lopez, Miriam Dorothy, "Bt-resistant European corn borers and *Nosema pyrausta*: implications for resistance management" (2008).  
*Retrospective Theses and Dissertations*. 14923.  
<https://lib.dr.iastate.edu/rtd/14923>

This Thesis is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact [digirep@iastate.edu](mailto:digirep@iastate.edu).

**Bt-resistant European corn borers and *Nosema pyrausta*:  
Implications for resistance management**

by

**Miriam Dorothy Lopez**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Entomology

Program of Study Committee:  
Leslie C. Lewis, Co-Major Professor  
Jon J. Tollefson, Co-Major Professor  
Douglas V. Sumerford  
Diane M. Debinski

Iowa State University

Ames, Iowa

2008

Copyright © Miriam Dorothy Lopez, 2008. All rights reserved.

UMI Number: 1453057



---

UMI Microform 1453057

Copyright 2008 by ProQuest Information and Learning Company.  
All rights reserved. This microform edition is protected against  
unauthorized copying under Title 17, United States Code.

---

ProQuest Information and Learning Company  
300 North Zeeb Road  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

## TABLE OF CONTENTS

LIST OF FIGURES	iv
LIST OF TABLES	vi
ABSTRACT	vii
CHAPTER 1. GENERAL INTRODUCTION	1
Thesis organization	1
Literature review	1
Rationale	5
Objectives	5
References	6
CHAPTER 2. <i>NOSEMA PYRAUSTA</i> AND CRY1AB-INCORPORATED DIET LED TO DECREASED SURVIVAL AND DEVELOPMENTAL DELAYS IN EUROPEAN CORN BORER	9
Abstract	9
Introduction	10
Materials and Methods	12
Results	15
Discussion	17
Figure Captions	21
Acknowledgements	28
References	28
CHAPTER 3. PROCLIVITY OF TRANSMISSION OF <i>NOSEMA PYRAUSTA</i> FROM MALE EUROPEAN CORN BORERS TO THEIR OFFSPRING	31
Abstract	31
Introduction	31
Materials and Methods	32
Results	34
Discussion	35
References	35
CHAPTER 4. EFFECTS OF INFECTION WITH <i>NOSEMA PYRAUSTA</i> ON SURVIVAL AND DEVELOPMENT OF OFFSPRING OF LABORATORY SELECTED BT-RESISTANT AND BT-SUSCEPTIBLE EUROPEAN CORN BORERS	37
Abstract	37
Introduction	38
Materials and Methods	40
Results	42
Discussion	43

Figure Captions	45
Acknowledgements	47
References	47
CHAPTER 5. ENCOUNTERING <i>NOSEMA PYRAUSTA</i> AND BT: EFFECTS ON LATE-INSTAR CRY1AB-RESISTANT AND SUSCEPTIBLE EUROPEAN CORN BORERS	50
Abstract	50
Introduction	51
Materials and Methods	53
Results	55
Discussion	57
Figure Captions	60
Acknowledgements	63
References	63
CHAPTER 6. GENERAL CONCLUSIONS	67
General Conclusions	67
Future Research Directions	69
References	70

## LIST OF FIGURES

### CHAPTER 2

Figure 1	Percentage survival of susceptible and resistant populations of <i>O. nubilalis</i> infected and not infected with <i>N. pyrausta</i> on diet incorporated with Cry1Ab and control diet.	22
Figure 2	Days from egg hatch to pupation of susceptible and resistant populations of <i>O. nubilalis</i> infected and not infected with <i>N. pyrausta</i> on diet incorporated with Cry1Ab and control diet.	23
Figure 3	Days from egg hatch to adult emergence of susceptible and resistant populations of <i>O. nubilalis</i> infected and not infected with <i>N. pyrausta</i> on diet incorporated with Cry1Ab and control diet.	24
Figure 4	Percentage survival at 14 days of resistant and susceptible <i>O. nubilalis</i> infected and not infected with <i>N. pyrausta</i> on control and 75% Bt tissue diets.	25
Figure 5	Mortality and survival by life stage at 14 days of resistant and susceptible <i>O. nubilalis</i> infected and not infected with <i>N. pyrausta</i> on control and 75% Bt tissue diets.	26
Figure 6	Stacked histogram showing pupation date of resistant and susceptible <i>O. nubilalis</i> infected and not infected with <i>N. pyrausta</i> on control or Cry1Ab-incorporated diet.	27

### CHAPTER 4

Figure 1	7 day survival of fully-resistant and partially-resistant <i>O. nubilalis</i> larvae infected and not infected with <i>N. pyrausta</i> on control diet, 3 ng/cm <sup>2</sup> Cry1Ab-overlaid diet and 30 ng/cm <sup>2</sup> Cry1Ab-overlaid diet.	46
Figure 2	Larval weight at 7 days of fully-resistant and partially-resistant <i>O. nubilalis</i> larvae infected and not infected with <i>N. pyrausta</i> on control diet, 3 ng/cm <sup>2</sup> Cry1Ab-overlaid diet and 30 ng/cm <sup>2</sup> Cry1Ab-overlaid diet.	47

### CHAPTER 5

- Figure 1 Percentage survival at 14 days of susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instar *O. nubilalis* transferred to Bt or regular diet treated or untreated with *N. pyrausta* spores. 61
- Figure 2 Change in weight at 7 days of susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instar *O. nubilalis* transferred to Bt or regular diet treated or untreated with *N. pyrausta* spores. 62
- Figure 3 Development as measured by the proportion of survivors that had pupated at 14 days of susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instar *O. nubilalis* transferred to Bt or regular diet treated or untreated with *N. pyrausta* spores. 63

## LIST OF TABLES

### CHAPTER 2

Table 1	Experimental parameters detailing genotype, infection status, exposure to Cry1Ab toxin or 75% Bt tissue incorporated diet, and data recorded.	15
---------	---	----

### CHAPTER 3

Table 1	Results of paired matings between a <i>N. pyrausta</i> -infected male and a presumed non-infected female	34
---------	--	----

### CHAPTER 4

Table 1	Test crosses	41
Table 2	Larval survival test of fixed effects	43
Table 3	Larval weight test of fixed effects	43

### CHAPTER 5

Table 1	Experimental treatments; numbers refer to treatment, all combinations of population, <i>N. pyrausta</i> , diet, and instar total 24 treatments.	54
Table 2	Analysis of Variance for three parameters: 14 day survival, 7 day change in weight, and proportion of survivors pupated at 14 days.	55

**ABSTRACT**

Interactions of *Nosema pyrausta* Paillot (Microsporida: Nosematidae) in *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), resistant and susceptible to the Bt protein Cry1Ab were studied. Differences in survival and developmental rate due to Cry1Ab exposure and *N. pyrausta* infection were quantified. Delayed development due to feeding on Cry1Ab has the potential to accelerate resistance evolution by precluding random mating. Resistant *O. nubilalis* infected with *N. pyrausta* were more susceptible to Cry1Ab than uninfected resistant *O. nubilalis*. Partially and fully resistant *N. pyrausta*-infected *O. nubilalis* exposed to Cry1Ab experienced reduced survival and weight compared with uninfected *O. nubilalis*. Encountering *N. pyrausta* in combination with Cry1Ab in late instars negatively impacted weight and development of resistant *O. nubilalis*, and killed susceptible *O. nubilalis*. These experiments illustrate the potential of *N. pyrausta* to influence resistance evolution by delaying development, and increasing susceptibility to Cry1Ab in resistant and susceptible *O. nubilalis*.

## CHAPTER 1. GENERAL INTRODUCTION

### THESIS ORGANIZATION

Chapter 1 is comprised of a general literature review of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), *Nosema pyrausta* Paillot (Microsporida: Nosematidae), *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Bt), Bt transgenic corn, and resistance management, and also includes rationale for the project and a statement of objectives. Chapters 2-5 will focus on the objectives investigated, followed by conclusions in chapter 6.

### LITERATURE REVIEW

***Ostrinia nubilalis***. The European corn borer, *Ostrinia nubilalis* (Hübner) is a member of the Crambidae family, order Lepidoptera. It is a holometabolous insect with five larval instars. Fifth instar *O. nubilalis* either pupate and emerge as adults in the same season (first generation in Iowa), or undergo diapause as a fifth instar, a period of suspended development (second generation in Iowa), pupating the following spring. In the northern United States and Canada, *O. nubilalis* is univoltine, or one generation per year. The central states *O. nubilalis* are bivoltine, and the southern states multivoltine, with 3-4 generations per year. *Ostrinia nubilalis* was introduced to the northeastern United States via broom corn imported from Hungary and Italy in the early 1900s (Caffery and Worthley, 1927), and spread south and west. Today, only 7 of the Western-most continental United States are free from *O. nubilalis* (Mason et al., 1996). Adults emerging from corn generally congregate in nearby grassy areas known as action sites to drink water and mate. Females emit a sex pheromone to attract males, normally mating on the second night following emergence. Females fly to corn to oviposit, returning to an action site, and remaining through the next day. Females produce an average of 2 egg masses per night for 10 nights following initial mating. The percentage of females that mate 2 or more times varies; reports have stated less than 5% (Mason et al., 1996) to up to 43% (Pesho, 1961). Egg masses containing an average of 15 eggs each are deposited mostly on the undersides of corn leaves, near the midrib. Hatch occurs approximately 3-

7 days later, and is dependent on temperature, humidity, weather conditions, and health of the adult.

First-generation, early-instar larvae feed in the whorl and on the mesophyll of leaves; later instars tunnel into the stalk. During the second generation, early instars travel to protected areas of the corn plant such as leaf axils or sheath collars, and may feed on pollen as well as leaf tissue. Later instars usually bore into the stalk or ear to feed, enter diapause, and remain there throughout the winter.

European corn borers feed on many host plants as larvae including agricultural commodities such as corn, peppers, snap beans, cotton, wheat, and potatoes. Corn, *Zea mays* L., is the main commodity attacked. *Ostrinia nubilalis* tunnel within the corn plants, causing disruption of the flow of water and nutrients leading to reduced ear and kernel size, broken stalks, and dropped ears. *Ostrinia nubilalis* also feed on ears causing direct yield loss. In the United States, annual costs associated with *O. nubilalis* in the form of yield loss and control measures in corn exceed 1 billion dollars (Mason et al., 1996). Control measures for *O. nubilalis* have included chemical and microbial insecticides, host plant resistance, biological control, and most recently, transgenically modified plants. Natural enemies of *O. nubilalis* include generalist predators such as ladybird beetles (adults and larvae), lacewing larvae and birds; parasitic insects including *Macrocentrus cingulum* Brischke, *Trichogramma brassicae* Bezdenko, *T. nubilale* Ertle & Davis, *T. ostrinae* Pang and Chen, and *Lydella thompsoni* Herting; and the pathogens *Nosema pyrausta* Paillot (Microsporida: Nosematidae) and *Beauveria bassiana* (Balsamo) Vuillemin. Many of these organisms have been introduced, augmented, conserved, or otherwise manipulated to manage *O. nubilalis*.

***Nosema pyrausta.*** *Nosema pyrausta* is a widespread indigenous microsporidium, and is an obligate intracellular parasite of *O. nubilalis* (Sajap and Lewis, 1988). In the corn ecosystem, *N. pyrausta* acts as a population regulator for *O. nubilalis* and it has been found to be density dependent, increasing with host density (Hill and Gary, 1979). *Nosema pyrausta* produces spores which are the infective unit. Infections are passed from insect to insect either from adult to egg transovarially (vertical transmission) or larva to larva by feeding on spore-contaminated frass in the immediate vicinity

(horizontal transmission). Effects of *N. pyrausta* vary and are dependent on intensity of infection. They include increased mortality in the larval and pupal stages, decreased adult longevity, and decreased fecundity (Zimmack and Brindley, 1957; Siegel et al., 1986; Windels et al., 1976). Infection can also prolong larval and pupal development in certain lower-temperature rearing conditions (Solter et al., 1990).

*Nosema pyrausta* spores, once ingested, extrude a polar filament which penetrates a midgut cell. This cell then produces additional spores that infect additional tissues. *Ostrinia nubilalis* tissues that are affected by *N. pyrausta* include midgut, fat body, malpighian tubules, silk glands, and reproductive tissues. In larval females, *N. pyrausta* can infect the epithelial, stroma, and germ cells of the reproductive tissues (Sajap and Lewis, 1988). This infection remains until adulthood, eventually leading to oviposition of transovarially infected eggs. Infection with *N. pyrausta* builds up over time in individuals. In a study by Bruck et al. (2001), a group of adult females had steadily increasing infection intensity over time, roughly 40 spores/mg at eclosion vs. 350 spores/mg 11 days later.

***Bacillus thuringiensis.*** *Bacillus thuringiensis* is a common, soil-dwelling, gram-positive bacterium with insecticidal properties. It was originally isolated in 1901 by Ishiwata from silkworm larvae, and then rediscovered in 1911 in a population of *Anagasta kuehniella* (Zeller) by Berliner, and was formally named at that time (Beegle and Yamamoto, 1992). *Bacillus thuringiensis* produces a crystalline toxin, or delta-endotoxin which disrupts the gut lining leading to cessation of feeding and eventual death from starvation or septicemia. The crystal must encounter specific conditions in the insect gut (i.e. alkaline pH) in order for the toxin to be solubilized and to bind. Because it acts directly on the gut lining, *B. thuringiensis* must be eaten to have activity. Different subspecies of *B. thuringiensis* have selective activity against certain groups of insects, for example *B. thuringiensis* subspecies *israelensis* affects Diptera, and subspecies *kurstaki* affects Lepidoptera.

The insecticidal properties of *B. thuringiensis* have led to it being formulated and incorporated as the active ingredient in insecticides; as early as 1938 a commercial product, Sporeine®, became available (Beegle and Yamamoto, 1992). This trend

continued, and today there are still Bt-based insecticides on the market including Dipel® and Thuricide®. These types of products are readily used in the forestry market, and also hold appeal for gardeners and organic farmers because of their specificity and natural active ingredient. Research into harnessing the insecticidal properties of *B. thuringiensis* continued, and in 1987 scientists first transformed tobacco and tomato plants to genetically express *B. thuringiensis* Cry proteins (Beegle and Yamamoto, 1992). In 1996, Bt corn expressing *B. thuringiensis* subspecies *kurstaki* protein was introduced commercially, and adoption rates have increased dramatically.

Bt corn hybrids are better protected from damage due to *O. nubilalis* than unmodified hybrids in years with *O. nubilalis* pressure. When a newly hatched larva feeds on tissues expressing the toxin, it quickly stops feeding due to gut paralysis and dies soon after, leaving the plant unharmed. Ideally, 100% control is achieved, however resistance to Bt sprays has been discovered in diamondback moth, *Plutella xylostella* L. (Tabashnik, 1994), and resistance to genetically-modified Bt crops has been demonstrated in laboratory colonies of other insects (Gould et al., 1995; Tabashnik, 1994) including *O. nubilalis* (Huang et al., 2005; Alves et al., 2006; Sumerford et al., 2008). While there have been no reports of field resistance to Bt crops, the possibility exists. A strategy implemented to prevent resistance development is the high dose/refuge strategy (USEPA 2001). The high dose/refuge strategy assumes that the level of Bt toxin expressed in the plant be high enough to kill 99.9% of individuals heterozygous for a resistance allele (“functionally recessive”), and that such alleles are rare in the overall population. The other requirement of this strategy is that a portion of the cropping area (currently 20% in the corn belt) be set aside and maintained as a refuge by planting it with a non-Bt form of the crop. The purpose of the refuge is to produce sufficient numbers of susceptible adults to mate with any potentially-resistant adults emerging from the Bt crop, therefore reducing the frequency of resistance alleles in the population and delaying the evolution of a resistant population. However, delays in larval development of resistant populations may lead to inconsistent timing of adult emergence and subsequent assortative mating among resistant adults.

## RATIONALE

The potential exists for *O. nubilalis* to develop resistance to Bt corn, and this project was undertaken to examine how infection with the microsporidium *N. pyrausta* may interact with the level of resistance to Bt in a Cry1Ab-resistant laboratory population of *O. nubilalis*. Studies on laboratory-selected resistant pink bollworm *Pectinophora gossypiella* (Saunders) have indicated that larval development can be slowed due to feeding on Bt cotton (Liu et al., 1999, 2001). If the same phenomenon is present in *O. nubilalis*, and resistant larvae develop more slowly on Bt corn than non-resistant larvae on unmodified corn, the likelihood of nonrandom or even preferential mating among resistant adults could increase, which has the potential to impact resistance evolution. There are studies that have shown an increased detrimental effect in *O. nubilalis* feeding on sources of Bt when infected with *N. pyrausta* (Pierce et al., 2001; Reardon et al., 2004). In addition it is known that infection with *N. pyrausta* can slow larval development. If resistant adults encounter *N. pyrausta*-infected adults and mate with them, their infected progeny may be less fit on Bt corn. Furthermore, it has been hypothesized that late-instar larvae are more able to withstand feeding on Bt and that movement from non-Bt to Bt plants by these larvae represents another avenue of resistance development (Huang et al., 1999, Walker et al., 2000; Reardon et al., 2007).

## OBJECTIVES

The following objectives were designed to examine the interactions between infection with *N. pyrausta* and resistance to Bt in *O. nubilalis*.

- Objective 1: Quantify developmental delays of Bt-resistant *O. nubilalis* larvae caused by feeding on Cry1Ab-incorporated diet; Quantify developmental delays in resistant and susceptible *O. nubilalis* larvae infected with *N. pyrausta*.
- Objective 2: Determine if adult male *O. nubilalis* can pass on *N. pyrausta* infection to offspring when mated to non-diseased females.
- Objectives 3 and 4: Examine survival and development of offspring of the following matings:
  - Non-infected resistant male mated with a *N. pyrausta*-infected susceptible female. Offspring will be heterozygous/*N. pyrausta*-infected

- Non-infected resistant male mated with a *N. pyrausta*-infected resistant female. Offspring will be homozygous/*N. pyrausta*-infected
- Objective 5: Quantify survival and development of *N. pyrausta*-infected larvae that have been moved as late-instars to Cry1Ab-incorporated diet from regular diet. Late instar (3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup>) *O. nubilalis* encountered *N. pyrausta* spores and Bt diet at the same time.

## REFERENCES

- Alves, A. P., T. A. Spencer, B. E. Tabashnik, and B. D. Siegfried. 2006.** Inheritance of resistance to the Cry1Ab *Bacillus thuringiensis* toxin in *Ostrinia nubilalis* (Lepidoptera: Crambidae). *J. Econ. Entomol.* 99: 494-501.
- Beegle, C. C., and T. Yamamoto. 1992.** Invitation paper (C.P. Alexander fund): history of *Bacillus thuringiensis* Berliner research and development. *Can Entomol.* 124: 587-616.
- Bruck, D. J., L. C. Lewis, and R. D. Gunnarson. 2001.** Interaction of *Nosema pyrausta* and temperature on *Ostrinia nubilalis* egg production and hatch. *J. Invertebr. Pathol.* 78: 210-214.
- Caffery, D. L., and L. H. Worthley. 1927.** A progress report on the investigation of the European corn borer. *USDA Bull.* 1476.
- Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995.** Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. *J. Econ. Entomol.* 88: 1545-1559.
- Hill, R.E., and W. J. Gary. 1979.** Effects of the microsporidium, *Nosema pyrausta*, on field populations of European corn borers in Nebraska. *Environ. Entomol.* 8: 91-95.
- Huang, F., L. L. Buschman, and R. A. Higgins. 2005.** Larval survival and development of susceptible and resistant *Ostrinia nubilalis* (Lepidoptera: Pyralidae) on diet containing *Bacillus thuringiensis*. *Agric. Forest Entomol.* 7: 45-52.
- Huang, F., L. L. Buschman, and R. A. Higgins. 1999.** Susceptibility of different instars of European corn borer (Lepidoptera: Crambidae) to diet containing *Bacillus thuringiensis*. *J. Econ. Entomol.* 92: 547-550.

- Liu, Y.B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, and A. C. Bartlett. 1999.** Development time and resistance to Bt crops. *Nature (Lond.)* 400: 519.
- Liu, Y. B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, M. A. Sims, S. K. Meyer, and Y. Carrière. 2001.** Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (Lepidoptera: Gelechiidae). *J. Econ. Entomol.* 94: 1237-1242.
- Mason, C. E., M. E. Rice, D. D. Calvin, J. W. Van Duyn, W. B. Showers, W. D. Hutchison, J. F. Witkowski, R. A. Higgins, D. W. Onstad, and G. P. Dively. 1996.** European corn borer ecology and management. North Central Regional Extension Pub. 327.
- Pesho, G. R. 1961.** Female mating patterns and spermatophore counts in the European corn borer, *Pyrausta nubilalis* (Hbn.). *Proceedings North Central Branch-ESA.* 16: 43.
- Pierce, C. M. F., L. F. Solter, and R. A. Weinzierl. 2001.** Interactions between *Nosema pyrausta* (Microsporidia: Nosematidae) and *Bacillus thuringiensis* subsp. *kurstaki* in the European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 94: 1361-1368.
- Reardon, B. J., R. L. Hellmich, D. V. Sumerford, and L. C. Lewis. 2004.** Growth, development, and survival of *Nosema pyrausta*-infected European corn borers (Lepidoptera: Crambidae) reared on meridic diet and Cry1Ab. *J. Econ. Entomol.* 97: 1198-1201.
- Reardon, K. T., R. L. Hellmich, D. V. Sumerford, L. C. Lewis, B. J. Reardon, and D. D. Calvin. 2007.** Influence of plant severing on movement of *Ostrinia nubilalis* larvae in *Zea mays* hybrid seed production fields. *J. Econ. Entomol.* 100: 1129-1135.
- Sajap, A. S., and L. C. Lewis. 1988.** Histopathology of transovarial transmission of *Nosema pyrausta* in the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 52: 147-153.
- Siegel J. P., J. V. Maddox, and W. G. Ruesink. 1986.** Lethal and sublethal effects of *Nosema pyrausta* on the European corn borer (*Ostrinia nubilalis*) in central Illinois. *J. Invertebr. Pathol.* 48: 167-173.
- Solter, L. F., D. W. Onstad, and J. V. Maddox. 1990.** Timing of disease-influenced processes in the life cycle of *Ostrinia nubilalis* infected with *Nosema pyrausta*. *J. Invertebr. Pathol.* 55: 337-341.

- Sumerford, D. V., L. C. Lewis, and J. C. Robbins. 2008.** Inheritance of resistance to Cry1Ab in a laboratory-selected colony of *Ostrinia nubilalis*: patterns of larval survival and larval development. Accepted to Heredity.
- Tabashnik, B. E. 1994.** Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39: 47-79.
- United States Environmental Protection Agency (US EPA). 2001.** Biopesticides registration action document – *Bacillus thuringiensis* plant-incorporated protectants. 16 October 2001. Content accessed 30 October 2007. [http://www.epa.gov/pesticides/biopesticides/pips/bt\\_brad.htm](http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm).
- Walker, K. A., R. L. Hellmich, and L. C. Lewis. 2000.** Late-instar European corn borer (Lepidoptera: Crambidae) tunneling and survival in transgenic corn hybrids. J. Econ. Entomol. 93: 1276-1285.
- Windels M. B., H. C. Chiang, and B. Furgala. 1976.** Effects of *Nosema pyrausta* on pupa and adult stages of the European corn borer *Ostrinia nubilalis*. J. Invertebr. Pathol. 27: 239-242.
- Zimmack, H. J., and T. A. Brindley. 1957.** Effect of the protozoan parasite *Perezia pyraustae* Paillot on the European corn borer. J. Econ. Entomol. 50: 637-640.

CHAPTER 2. *NOSEMA PYRAUSTA* AND CRY1AB-INCORPORATED  
DIET LED TO DECREASED SURVIVAL AND DEVELOPMENTAL  
DELAYS IN EUROPEAN CORN BORER

A paper to be submitted to J. Econ. Entomol.

Miriam D. Lopez<sup>1,2</sup>, Douglas V. Sumerford<sup>1</sup>, Leslie C. Lewis<sup>1</sup>

**ABSTRACT**

The high dose/refuge strategy for delaying evolution of resistance to Bt corn relies on random mating between heterozygous-resistant European corn borers, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), and susceptible *O. nubilalis* from the refuge. However, differences in developmental rate due to feeding on Bt corn, or infection with the microsporidium *Nosema pyrausta* Paillot (Microsporida: Nosematidae) may result in assortative mating. Developmental delays and mortality caused by infection with *N. pyrausta* and feeding on Bt were quantified alone and in combination in Cry1Ab-resistant and susceptible *O. nubilalis*. Feeding on Cry1Ab-incorporated diet significantly increased number of days from hatch to pupation and decreased survival in the resistant population. Infection with *N. pyrausta* increased mortality and lengthened development in both the resistant and susceptible populations. The combination of Cry1Ab-incorporated diet and infection with *N. pyrausta* in resistant *O. nubilalis* increased days from hatch to pupation and mortality to a greater extent than either factor alone. Longer larval periods of resistant *O. nubilalis* on Bt corn could lead to temporal isolation from refuge *O. nubilalis*, and therefore assortative mating, which would hasten evolution of resistance. Developmental delays due to infection with *N. pyrausta* may increase the likelihood of mating between resistant and infected refuge adults, producing infected offspring which are more susceptible to Bt.

---

<sup>1</sup> Graduate student, Adjunct Assistant Professor, Adjunct Professor, respectively, Department of Entomology, Iowa State University. USDA-ARS, Ames, Iowa.

<sup>2</sup> Primary researcher and author

## INTRODUCTION

European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) was introduced into the northeastern United States via broom corn imported from Hungary and Italy in the early 1900s (Caffery and Worthley, 1927), and spread south and west. Today, only 7 of the continental United States are free from *O. nubilalis* (Mason et al., 1996). *Ostrinia nubilalis* is a serious pest, inflicting average yield losses of 5% annually, making it the second most damaging pest of corn in Iowa (Bergman et al., 1985). European corn borer larvae damage plants by tunneling in stalks, which leads to reduced ear and kernel size as well as dropped ears and broken stalks; larvae also directly damage ears. Various methods of control have been employed to combat this pest including habitat destruction, chemical and microbial insecticides, host plant resistance, biological control, and recently transgenically-modified corn hybrids.

One organism identified as a biocontrol agent of *O. nubilalis* is the microsporidium *Nosema pyrausta* Paillot (Microsporida: Nosematidae). *Nosema pyrausta* is an obligate parasite of *O. nubilalis*, and infection with *N. pyrausta* causes decreased fecundity and survival. This pathogen is widespread and has been shown to delay larval development and contribute to mortality (Zimmack and Brindley, 1957; Solter et al., 1990b; Sajap and Lewis, 1992). *Ostrinia nubilalis* infected with *N. pyrausta* also have reduced fecundity (Siegel et al., 1986; Windels et al., 1976) compared to non-infected *O. nubilalis*. *Nosema pyrausta* is transmitted from insect to insect transovarially (Sajap and Lewis, 1988; 1992), as well as horizontally, by feeding on spores deposited in frass (Andreadis, 1986; 1987). While *N. pyrausta* will not provide complete suppression of *O. nubilalis* for crop protection, the sustained chronic effects of infection can act as a population regulator (Onstad and Maddox, 1989).

To combat the corn borer, molecular biologists have modified corn hybrids to produce an insecticidal toxin derived from the bacterium *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Bt). These transgenic corn hybrids provide protection from *O. nubilalis* damage with benefits including fewer chemical inputs to the environment, and season-long effective control. One concern involving the planting of Bt corn is the possibility of *O. nubilalis* evolving resistance and rendering the technology less effective.

In order to delay resistance evolution, the high dose/refuge strategy is employed (US EPA 2001). For the high dose strategy to be effective, levels of Cry toxin produced in the plant must be great enough to kill 99.9% of individuals heterozygous for a resistance allele (“functionally recessive”), and such alleles must be rare in the population. In addition a specified portion (currently 20% in the corn belt) of the corn acreage be planted to non-genetically modified corn as a refuge. The purpose of the refuge corn is to produce sufficient numbers of susceptible *O. nubilalis* to lower the likelihood of matings among heterozygous-resistant *O. nubilalis*. The greater probability of matings between heterozygous-resistant *O. nubilalis* and susceptible *O. nubilalis* will reduce the likelihood of producing homozygous-resistant *O. nubilalis* and therefore, delay or eliminate the evolution of resistance to Bt crops. The refuge may also act as a reserve for natural enemies of the target pest (Bruck et al., 2007).

One assumption of the high dose/refuge strategy is heterozygous-resistant individuals will randomly mate with susceptible *O. nubilalis* adults emerging from the refuge. For this to occur adult emergence inside and outside the refuge must occur at comparable times. However, resistant laboratory-selected populations of pink bollworm *Pectinophora gossypiella* (Saunders) exhibit increased developmental periods on Bt cotton and Cry1Ac-incorporated artificial diets compared with susceptible *P. gossypiella* on non-Bt cotton and diet (Liu et al., 1999; 2001). If this same phenomenon of delayed development occurs in resistant *O. nubilalis* on Bt corn, it could lead to asynchronous adult emergence relative to refuge moths, and assortative mating of resistant adults. Another ecological factor that delays larval and pupal development of *O. nubilalis* is infection with *N. pyrausta* (Solter et al., 1990b). *Nosema pyrausta* is ubiquitous in *O. nubilalis* populations, and could also lead to asynchronous adult emergence of uninfected relative to *N. pyrausta*-infected moths.

The objectives of this study were 1) to examine survival of susceptible and resistant *O. nubilalis* phenotypes infected and not infected with *N. pyrausta* on Cry1Ab-incorporated and control diets; 2) to measure the individual development of susceptible and resistant *O. nubilalis* phenotypes infected and not infected with *N. pyrausta* on Cry1Ab-incorporated and control diets; and 3) to examine the effect of infection with *N.*

*pyrausta* on survival of susceptible and resistant *O. nubilalis* feeding on tissue diet containing Cry1Ab.

## MATERIALS AND METHODS

### Experiment 1: Development and survival on Cry1Ab-incorporated diet.

This experiment was designed to examine length of developmental period and survival of *N. pyrausta*-infected and uninfected *O. nubilalis* larvae originating from Cry1Ab-susceptible and resistant colonies feeding on control and Cry1Ab-incorporated diets. Neonates were placed onto diet and examined daily for pupation, adult emergence, or death. Infection status was recorded at death or adult emergence. Six treatments were comprised of combinations of colony, *N. pyrausta* status, and diet (See Table 1). Susceptible *O. nubilalis* on Cry1Ab-incorporated diet treatments were not included because previous experimentation showed there would be no survival.

**Insect Preparation.** Populations of *O. nubilalis* resistant to high levels of Cry1Ab toxin have been selected for and maintained in the laboratory >50 generations (Sumerford personal comm., 2008). The Cry1Ab-resistant population is > 2500x resistant. Eggs from this population were divided into two groups and hatched in containers (25 cm diameter) (Pioneer Plastics, Inc., Dixon, KY) containing 930 g meridic diet (Guthrie et al., 1985) without Fumidil B, which inhibits spore production of *N. pyrausta* (Lewis and Lynch, 1970). To generate a highly infected population of resistant *O. nubilalis*, half of the diet containers, each containing approximately 600 larvae were inoculated with spores of *N. pyrausta* at a rate of 1000 spores/mm<sup>2</sup> of diet approximately 4 days after egg hatch. Because *O. nubilalis* larvae can obtain infection with *N. pyrausta* by consuming spore-containing frass, (horizontal transmission), communal rearing in an arena without Fumidil B facilitates spread of *N. pyrausta* within the container. This method of inoculation led to a high percentage of infection.

Susceptible populations were produced in the same way: a research population of non-infected *O. nubilalis* is continually reared at the USDA-ARS, Corn Insects and Crop Genetics Research Unit. Eggs from this population were divided into two groups, non-infected and infected. The “infected” population was inoculated with spores as described above to ensure a high rate of infection. Adults from all 4 populations were maintained

in ideal mating and oviposition conditions in an environmental chamber. Eggs from these populations were either non-infected or vertically infected, and were used in temporal block 1. The same procedures were followed to generate vertically infected and non-infected populations of *O. nubilalis* for temporal block 2.

**Diet preparation.** Experiment 1 treatments consisted of populations of *O. nubilalis* on meridic diet with or without Cry1Ab toxin incorporated. Cry1Ab toxin (trypsin-activated Cry1Ab; obtained from Dr. M. Carey, Dept. of Biochemistry, Case Western Reserve University, Cleveland, OH) was incorporated at a concentration of 3 µg/ml diet. The concentration of 3 µg/ml was chosen to provide a conservative test of effects. Diets were prepared according to procedures found in Guthrie et al. (1965), incorporated with Cry1Ab toxin, and poured into 18 ml plastic cups (Anderson Tool and Die, Linden, NJ) to solidify. Neonatal larvae (<24 hr old) were introduced to individual diet cups and covered with unwaxed cardboard lids (Stanpac, Lewiston, NY).

**Data collection.** Experiment 1 larvae were examined daily and data were recorded on mortality, pupation, and emergence. Approximately one day after pupal formation, pupae were transferred to empty 18.5 ml plastic cups to emerge. Instar and infection status were determined immediately for any insects that died in the larval stage. Cadavers were either prepared as a squash mount on a microscope slide in a drop of water and examined, or, if larger than 2<sup>nd</sup> instar, homogenized in a tissue grinder with water and the resulting liquid examined using a light microscope (400X), and given a designation of plus or minus for *N. pyrausta* infection. Infection status for emerged adults was determined as follows: 1-2 drops of distilled water were added to the meconium, and mixed together (Inglis et al., 2003). The resulting solution was examined on a slide as described above.

**Experimental design and analysis.** Experiment 1 consisted of 2 temporal blocks each with six treatments replicated 10 times. A tray of 30 cups was one replication, with the six treatments randomly assigned to a row within the tray. One replication included 5 larvae per treatment for a total of 600 individuals. To enhance normality and homogenize variances, developmental data were Log<sub>10</sub>-transformed. Survival estimates and developmental data were analyzed by Proc Mixed of SAS (v. 9.1.3) using a mixed

model ANOVA. Treatments were considered fixed effects, and temporal blocks and replications within blocks were considered random sources of variance. Significant treatment effects were followed by pairwise comparisons of least-squares means via the Tukey Kramer method ( $P < 0.05$ ).

### **Experiment 2: Survival on Bt tissue diet.**

This follow-up experiment was designed to observe the effects of feeding on 75% Bt tissue diet on susceptible and resistant *O. nubilalis* infected and not infected with *N. pyrausta*, and confirm that the effects of *N. pyrausta* on Bt tissue diet would mirror those on Cry1Ab-incorporated diet. This study tested the same populations on a more realistic growth medium, with the addition of susceptible populations exposed to Bt. Neonates were placed onto tissue diet and examined at 14 days. Data were recorded on mortality and larval instar or life stage of survivors. Individual infection status was not recorded. Eight treatments were comprised of combinations of colony, *N. pyrausta* status, and diet (See Table 1).

**Insect Preparation.** Populations of *N. pyrausta*-infected and non-infected susceptible and resistant *O. nubilalis* were established according to the procedures outlined in experiment 1. Because it is difficult to establish and synchronize the four populations, limited numbers of larvae were available for experimentation.

**Diet preparation.** Freeze-dried corn tissue expressing the Cry1Ab toxin was reconstituted and substituted for 75% by weight of the nutritive ingredients (dextrose, casein, beta sitosterol, salt mix #2, vitamin supplement, and ascorbic acid) in meridic diet; see Wilson and Wissink (1986) for tissue substitution method with the exception that all wheat germ was eliminated. The diet was poured onto a plate to a thickness of approximately 1 cm and allowed to solidify. Cylindrical plugs were then removed from the diet slab and placed into opaque 128 cell Bio-Assay trays (Bio-serv, Frenchtown, NJ). Controls fed on wheat-germ based meridic diet poured directly into Bio-Assay tray cells. Neither diet contained Fumidil B. Neonatal larvae of the appropriate population were placed onto individual diet plugs, and trays were sealed with 16-cell self-adhesive covers (Bio-serv) which allowed for air and moisture exchange while preventing larval movement between cells.

**Experimental design.** Experiment 2 consisted of all genotype/infection/diet combinations. Each set of 32 diet cells was comprised of 20 Bt tissue cells, and 12 non-Bt cells, and was assigned to a genotype/infection treatment. Due to limitations in the number of larvae and the amount of tissue diet available, the number of sets infested was not equal among populations (See Table 1). Fewer susceptible clean and diseased individuals were infested due to the lower variance in their response to Bt observed during experiment 1.

**Data collection and analysis.** Experiment 2 larvae were examined at 14 days, data were recorded on mortality, and instar or life stage of survivors.

**Table 1: Experimental parameters detailing genotype, infection status, exposure to Cry1Ab toxin or Bt tissue incorporated diets, and data recorded.**

<i>Experiment 1</i>					
Treatment	Genotype	Infection	Diet	Data	# Individuals
1	Susceptible	non-infected	Control	Mortality, Development	100
2	Susceptible	<i>N. pyrausta</i>	Control	Mortality, Development	100
3	Resistant	non-infected	Control	Mortality, Development	100
4	Resistant	non-infected	Cry1Ab toxin	Mortality, Development	100
5	Resistant	<i>N. pyrausta</i>	Control	Mortality, Development	100
6	Resistant	<i>N. pyrausta</i>	Cry1Ab toxin	Mortality, Development	100
<i>Experiment 2</i>					
1	Susceptible	non-infected	Control	Mortality, Instar at 14 d	12
2	Susceptible	non-infected	75% Bt tissue	Mortality, Instar at 14 d	20
3	Susceptible	<i>N. pyrausta</i>	Control	Mortality, Instar at 14 d	12
4	Susceptible	<i>N. pyrausta</i>	75% Bt tissue	Mortality, Instar at 14 d	20
5	Resistant	non-infected	Control	Mortality, Instar at 14 d	60
6	Resistant	non-infected	75% Bt tissue	Mortality, Instar at 14 d	100
7	Resistant	<i>N. pyrausta</i>	Control	Mortality, Instar at 14 d	60
8	Resistant	<i>N. pyrausta</i>	75% Bt tissue	Mortality, Instar at 14 d	100

## RESULTS

**Experiment 1.** Infection with *N. pyrausta* significantly decreased survival to adulthood, as did feeding on Cry1Ab-incorporated diet (Fig. 1). The treatments were significantly different ( $F=57.40$ ;  $df=5, 91$ ;  $P<.0001$ ). Larvae from the susceptible colony that were infected with *N. pyrausta* (trt. 2) had significantly lower survival than non-infected susceptible larvae (trt. 1). The same trend was observed in the resistant larvae feeding on non-Bt diet (trt. 5 survival significantly lower than trt. 3). Feeding on

Cry1Ab-incorporated diet significantly decreased survival of uninfected resistant *O. nubilalis* (comparison of trts. 3 and 4). The combination of feeding on Cry1Ab-incorporated diet and infection with *N. pyrausta* (trt. 6) led to lower survival than either factor alone.

Larval development, measured by days from hatch to pupation, was significantly increased by infection with *N. pyrausta*, and also by feeding on Cry1Ab-incorporated diet (Fig. 2). Estimates of larval development were analyzed by ANOVA followed by pairwise comparisons of least-squares means via the Tukey Kramer method ( $P < 0.05$ ). The overall treatment effect was significant ( $F = 98.62$ ;  $df = 5, 77$ ;  $P < .0001$ ). Infection with *N. pyrausta* significantly increased the number of days spent in the larval stage for both the susceptible populations (trts. 1 vs. 2), and the resistant populations (trts. 3 vs. 5). Feeding on Cry1Ab also resulted in significantly longer larval stages in the resistant, uninfected population (trt. 4). The combined effects of feeding on Cry1Ab-incorporated diet, and infection with *N. pyrausta* increased developmental period further than either factor alone (trt. 6).

Total developmental period (days from egg hatch to adult emergence) was increased due to both infection with *N. pyrausta*, and feeding on Cry1Ab-incorporated diet (Fig. 3). Estimates of hatch to emergence period were analyzed by ANOVA with further least-squares means comparisons, Method=Tukey Kramer ( $P < 0.05$ ). ANOVA analysis showed that overall treatments were different ( $F = 101.35$ ;  $df = 5, 66$ ;  $P < .0001$ ). Infection with *N. pyrausta* significantly increased time from hatch to emergence in both the susceptible (trts. 1 vs. 2) and resistant (trts. 3 vs. 5) populations on non-Bt diet. Feeding on Cry1Ab diet increased total developmental period in non-infected resistant *O. nubilalis* (trt. 4) compared to non-infected on non-Bt diet (trt. 3). The combination of infection with *N. pyrausta* and feeding on Cry1Ab-incorporated diet (trt. 6) did not significantly increase developmental period compared with *N. pyrausta* alone (trt. 5), or Cry1Ab-incorporated diet alone (trt. 4). However, loss of many observations due to mortality of pupae decreased the statistical power to detect significant differences.

**Experiment 2.** Feeding on Bt tissue diet was detrimental to survival of both resistant and susceptible *O. nubilalis* genotypes (Fig. 4). At 14 days, no susceptible

larvae had survived feeding on Bt tissue diet (trts. 2 and 4). Some resistant larvae (38%) were able to survive feeding on Bt tissue diet. When *N. pyrausta*-infected resistant larvae fed on Bt tissue diet, survival was extremely low, 2.02%, and was not significantly different from the susceptible/Bt tissue diet treatments 2 and 4. Feeding on Bt tissue diet also led to developmental delays in the resistant population (trt. 6) (Fig. 5).

## DISCUSSION

Both feeding on Cry1Ab-incorporated diet and infection with *N. pyrausta* caused developmental delays in larval *O. nubilalis*. There was a significant delay in time to pupation in resistant *O. nubilalis* on Cry1Ab-incorporated diet compared with susceptible *O. nubilalis* on control diet (25 vs. 17 d). There was also a significant delay in *N. pyrausta*-infected *O. nubilalis* compared with non-infected *O. nubilalis* on non-Cry1Ab diet (23 vs. 17 d). For the high dose/refuge strategy of resistance management to effectively slow resistance evolution it is imperative that resistant individuals mate randomly, or preferentially with susceptible individuals emerging from the refuge. The prolonged larval stage experienced by the non-infected resistant population feeding on Cry1Ab-incorporated diet caused a delay in total developmental time relative to the susceptible non-infected population. By the time resistant *O. nubilalis* feeding on Cry1Ab-incorporated diet began to pupate, the large majority of susceptible *O. nubilalis* feeding on non-Bt diet had already pupated (Fig. 6). If similar patterns occur under field conditions, by the time resistant *O. nubilalis* are emerging from Bt corn in the field, their refuge counterparts would have already emerged and begun mating and ovipositing, resulting in assortative mating. According to a study by Fadamiro and Baker (1999), females that were delayed in first mating by 3 and 7 days experienced significantly reduced and near-zero fecundity, respectively, compared to their non-delayed counterparts. In addition to females, timing of mating also affects male reproductive success. Royer and McNeil (1993) found that the size of male spermatophores decreased with successive matings, which led to decreases in both the number and fertility of eggs from matings with 2<sup>nd</sup> and later females. These works illustrate two potential impediments to production of offspring between temporally-shifted populations as would be the case when prolonged development in resistant *O. nubilalis* leads to delayed mating

with refuge adults, or even reproductive isolation. If by the time the ‘resistant’ moths have emerged there are no more ‘susceptible’ moths to mate with, assortative mating would increase the production of homozygous-resistant individuals, and therefore increase the rate of resistance evolution. Delays in mating between these populations could also inhibit production of heterozygous-resistant offspring. These types of responses to feeding on Bt corn would reduce or eliminate the effectiveness of the refuge in delaying resistance evolution. Reports have been made regarding the spatial scale of gene flow between refuge and resistant *O. nubilalis* (Dalecky et al., 2006), but temporal scale differences due to developmental delays and subsequent effects on gene flow in *O. nubilalis* have not been studied. First-generation female *O. nubilalis* generally emerge, move to action sites, and mate on the second night. Reports on the percentage of females that mate more than once vary from less than 5% (Mason et al., 1996) to up to 43% (Pesho, 1961). Females deposit an average of 2 egg masses per night for up to 10 nights, but the majority are deposited in the first 6 nights (Mason et al., 1996). If resistant and susceptible populations are completely temporally isolated, or have only minor overlapping periods, the refuge will not bring about random matings between resistant and susceptible genotypes.

Another ecological factor affecting developmental periods to consider is infection with *N. pyrausta*. Results from this study mirror others in showing that infection with *N. pyrausta* can lead to prolonged larval and pupal stages (Solter et al., 1990b). Assuming the developmental delays evident in this experiment are representative of field situations, resistant adults will be more temporally aligned with *N. pyrausta*-infected refuge adults than uninfected refuge adults (Fig. 6). In years where the percentage of *O. nubilalis* infected with *N. pyrausta* is high, resistant adults emerging from Bt corn may be more likely to encounter *N. pyrausta*-infected adults than non-infected adults because both resistant *O. nubilalis* in Bt corn, and infected *O. nubilalis* in the refuge will be delayed relative to non-infected refuge *O. nubilalis*. Because *N. pyrausta* is transovarially transmitted, resistant adults mating with infected susceptible partners will produce some infected offspring. *Ostrinia nubilalis* infected with *N. pyrausta* on non-Bt diet began emerging before resistant, non-infected *O. nubilalis* on Cry1Ab-incorporated diet. Male

*O. nubilalis* are known to emerge before females (Caffery and Worthley, 1927), which would make a mating between an infected susceptible female and a non-infected resistant male more likely than a mating between a resistant non-infected female and an infected susceptible male. Infected females can transmit *N. pyrausta* to some or all of their offspring, and while the possibility for males to do likewise has been postulated, there is little evidence it would contribute to spread of the disease (Solter et al., 1990a). Mating between infected susceptible females and non-infected resistant males would produce offspring partially resistant to Bt, but also potentially infected with *N. pyrausta*. More research needs to be conducted to determine what effect *N. pyrausta* infection will have on the heterozygous offspring of a resistant moth mated with a susceptible moth.

*Nosema pyrausta* is known to cause greater mortality in *O. nubilalis* fed Bt-incorporated diets when compared with non-infected *O. nubilalis* (Pierce et al., 2001; Reardon et al., 2004), but its effects on Bt-resistant *O. nubilalis* have not been examined. This study demonstrates increased mortality of resistant *O. nubilalis* infected with *N. pyrausta* on Cry1Ab-incorporated diet compared with uninfected *O. nubilalis*. While both *N. pyrausta* and Cry1Ab-incorporated diet caused a decrease in survival to adulthood, the combination of infection with *N. pyrausta* and exposure to Cry1Ab-incorporated diet caused extremely high mortality, 96%. If potentially-resistant *O. nubilalis* in the field respond similarly to the combined influences of infection with *N. pyrausta* and feeding on Bt corn, the chances of infected resistant *O. nubilalis* surviving on Bt corn to confer resistance to the next generation would be drastically reduced. *Nosema pyrausta* is a commonly-occurring pathogen of *O. nubilalis* that functions as a natural population regulator, and where resistance management is concerned, the effects of feeding on Bt diet are magnified when infection with *N. pyrausta* is present.

The follow-up study tested whether the same trends would be apparent on 75% Bt tissue diet. In this experiment, infection with *N. pyrausta* caused genotypically resistant *O. nubilalis* to have the same response to feeding on 75% Bt tissue diet as susceptible *O. nubilalis*. Resistant *O. nubilalis* also experienced developmental delays when feeding on 75% Bt tissue diet, which mirrored the results of experiment 1. According to a study by Solter et al. (1990a), over 60% of *O. nubilalis* larvae transovarially infected with *N.*

*pyrausta* died prior to pupating. A study by Siegel et al. (1986) also showed that *N. pyrausta* can cause severe reductions in survival to adulthood of transovarially-infected *O. nubilalis*. Transovarially-infected *O. nubilalis* experience decreased survival compared with their uninfected counterpart, and in the current experiment, when Bt-resistant transovarially-infected larvae were challenged with 75% Bt tissue diet, survival was nearly zero, essentially eliminating their resistance. If infection with *N. pyrausta* negates resistance it may act not only as a natural population regulator of *O. nubilalis*, but also as a resistance mitigator, by rendering resistant *O. nubilalis* susceptible to feeding on Bt corn. Infection with *N. pyrausta* could compromise the ability of resistant *O. nubilalis* to survive to adulthood on Bt corn, and therefore slow down the rate of resistance evolution in the field. The potential of a virus to slow the evolution of resistance to Bt in diamondback moth *P. xylostella* L. has been investigated (Raymond et al., 2007), but the potential of microsporidia to do likewise in *O. nubilalis* has not.

The refuge requirement for resistance management serves to produce sufficient numbers of Bt-susceptible *O. nubilalis* to mate with resistant *O. nubilalis* and reduce the frequency of genes conferring resistance; however, temporal isolation due to delayed development could negate this function. *Ostrinia nubilalis* living in refuge corn may provide a source of *N. pyrausta*, which has been shown to significantly decrease survival of resistant *O. nubilalis* on Cry1Ab-incorporated diet and Bt tissue-incorporated diets. Because *N. pyrausta* infection can also delay larval development, resistant adults may be more likely to mate with similarly-delayed *N. pyrausta*-infected refuge adults than uninfected refuge adults, which may influence resistance evolution. Additional studies need to be conducted including flight capabilities and mating behaviors of *N. pyrausta*-infected adults before they can be considered mitigators of *O. nubilalis* resistance to Bt corn, but the potential exists. At the very least, attempts should be made to conserve this natural enemy of *O. nubilalis* through refuge planting and no-till cultivation.

## FIGURE CAPTIONS

- Figure 1. Percentage survival of susceptible (S) and resistant (R) populations of *O. nubilalis* infected with *N. pyrausta* (Np +) and not infected with *N. pyrausta* (Np -) on diet incorporated with Cry1Ab (Bt) and control diet (Reg). Least-squares means comparison: Method=Tukey-Kramer ( $P<0.05$ ) used to generate estimates. Survival estimates labeled with the same letter are not significantly different at the P .05 level.
- Figure 2. Days from egg hatch to pupation [ $\text{Log}_{10}$ -transformed] of susceptible (S) and resistant (R) populations of *O. nubilalis* infected with *N. pyrausta* (Np +) and not infected with *N. pyrausta* (Np -) on diet incorporated with Cry1Ab (Bt) and control diet (Reg). Least-squares means comparison: Method=Tukey-Kramer ( $P<0.05$ ) used to generate estimates. Estimates followed by the same letter are not significantly different.
- Figure 3. Days from egg hatch to adult emergence [ $\text{Log}_{10}$ -transformed] of susceptible (S) and resistant (R) populations of *O. nubilalis* infected with *N. pyrausta* (Np +) and not infected with *N. pyrausta* (Np -) on diet incorporated with Cry1Ab (Bt) and control diet (Reg). Least-squares means comparison: Method=Tukey-Kramer ( $P<0.05$ ) used to generate estimates. Estimates followed by the same letter are not significantly different.
- Figure 4. Percentage survival at 14 days of resistant (R) and susceptible (S) *O. nubilalis* infected (Np +) and not infected (Np -) with *N. pyrausta* on control (Reg) and 75% Bt tissue (Bt) diets.
- Figure 5. Mortality and survival by life stage at 14 days of resistant (R) and susceptible (S) *O. nubilalis* infected (Np +) and not infected (Np -) with *N. pyrausta* on control (Reg) and 75% Bt tissue (Bt) diets.
- Figure 6. Stacked histogram showing pupation date [ $\text{Log}_{10}$ -transformed] of resistant (R) and susceptible (S) *O. nubilalis* infected (Np +) and not infected (Np -) with *N. pyrausta* on control (Reg) or Cry1Ab-incorporated (Bt) diet.

Fig. 1

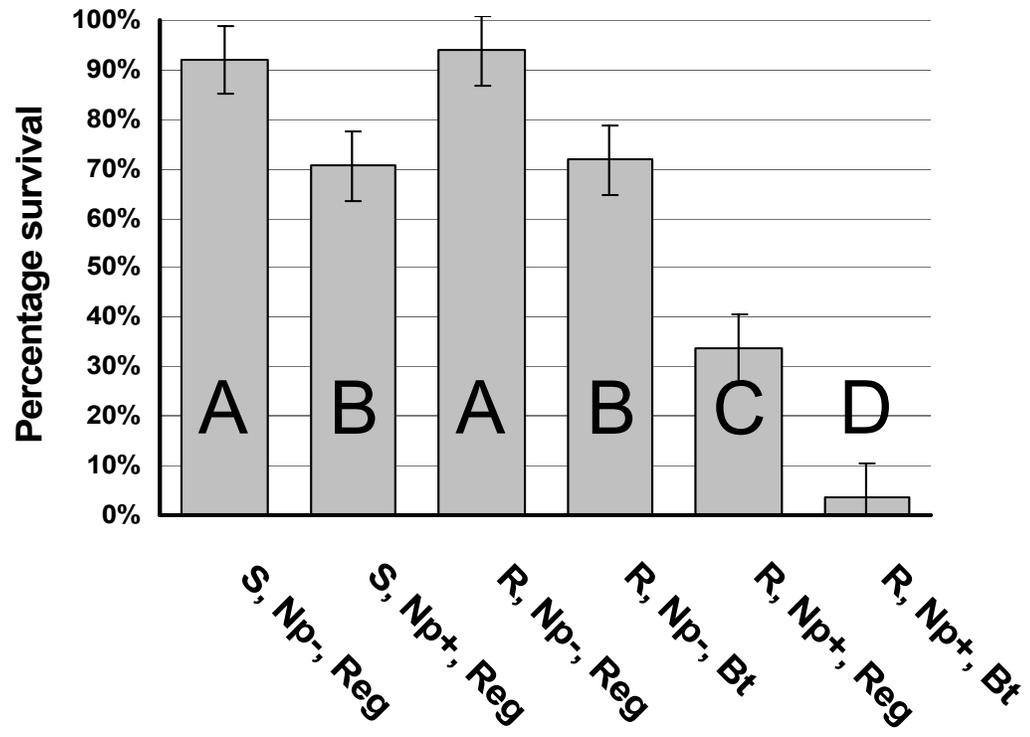


Fig. 2

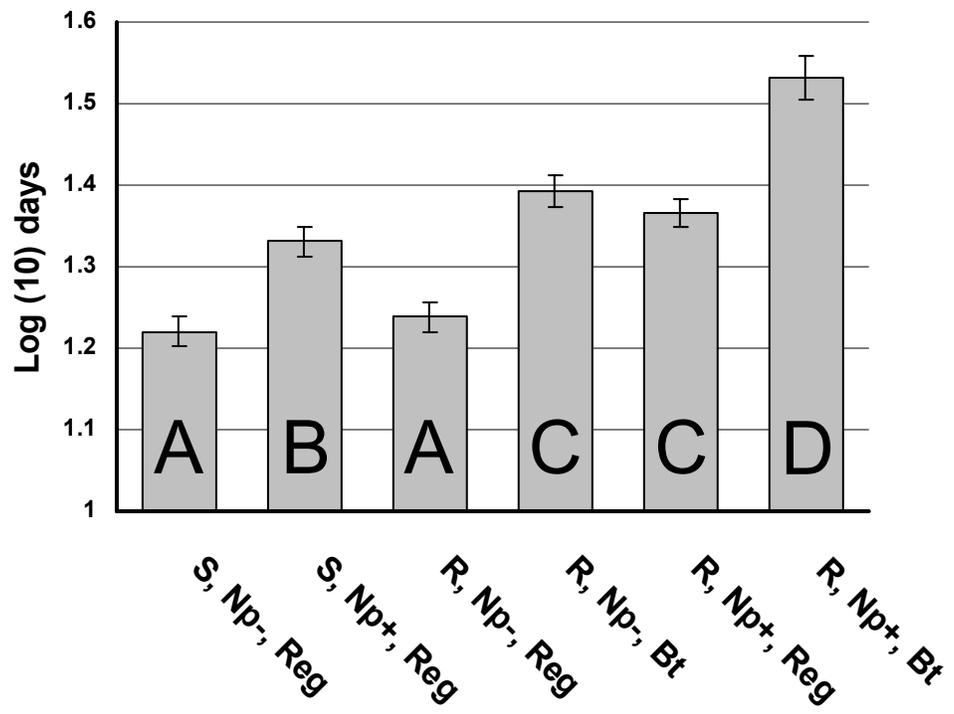


Fig. 3

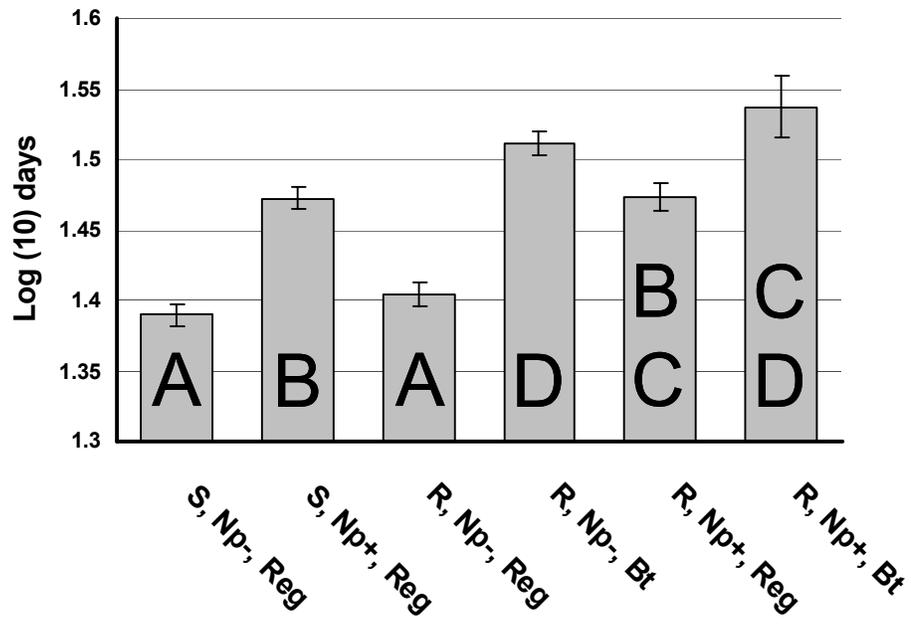


Fig. 4

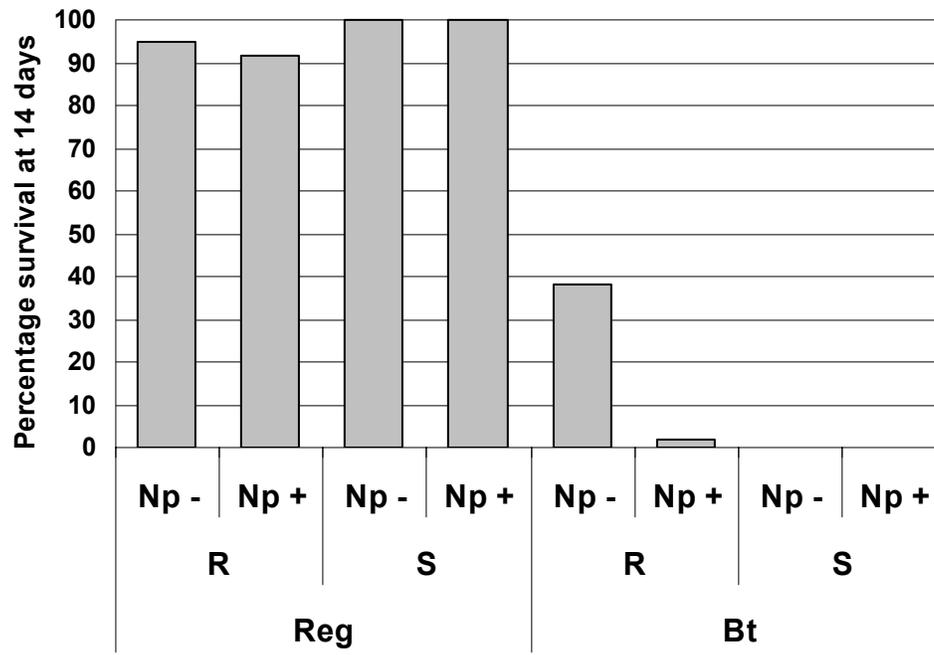


Fig. 5

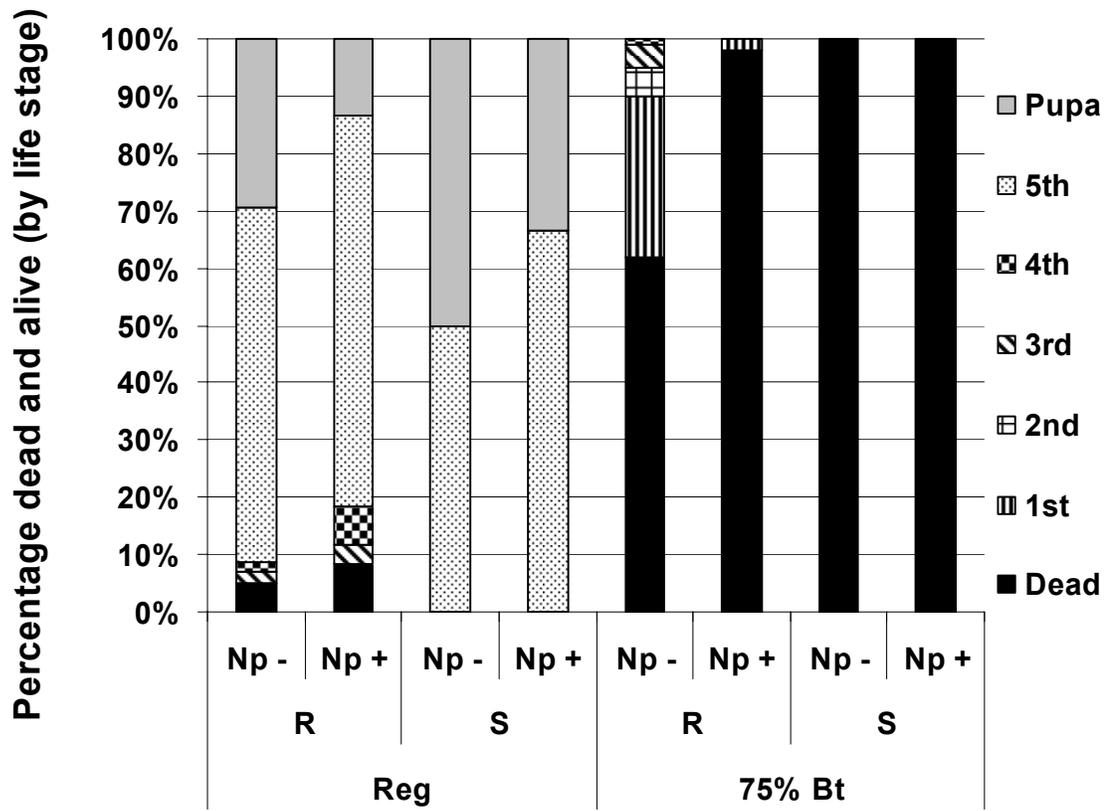
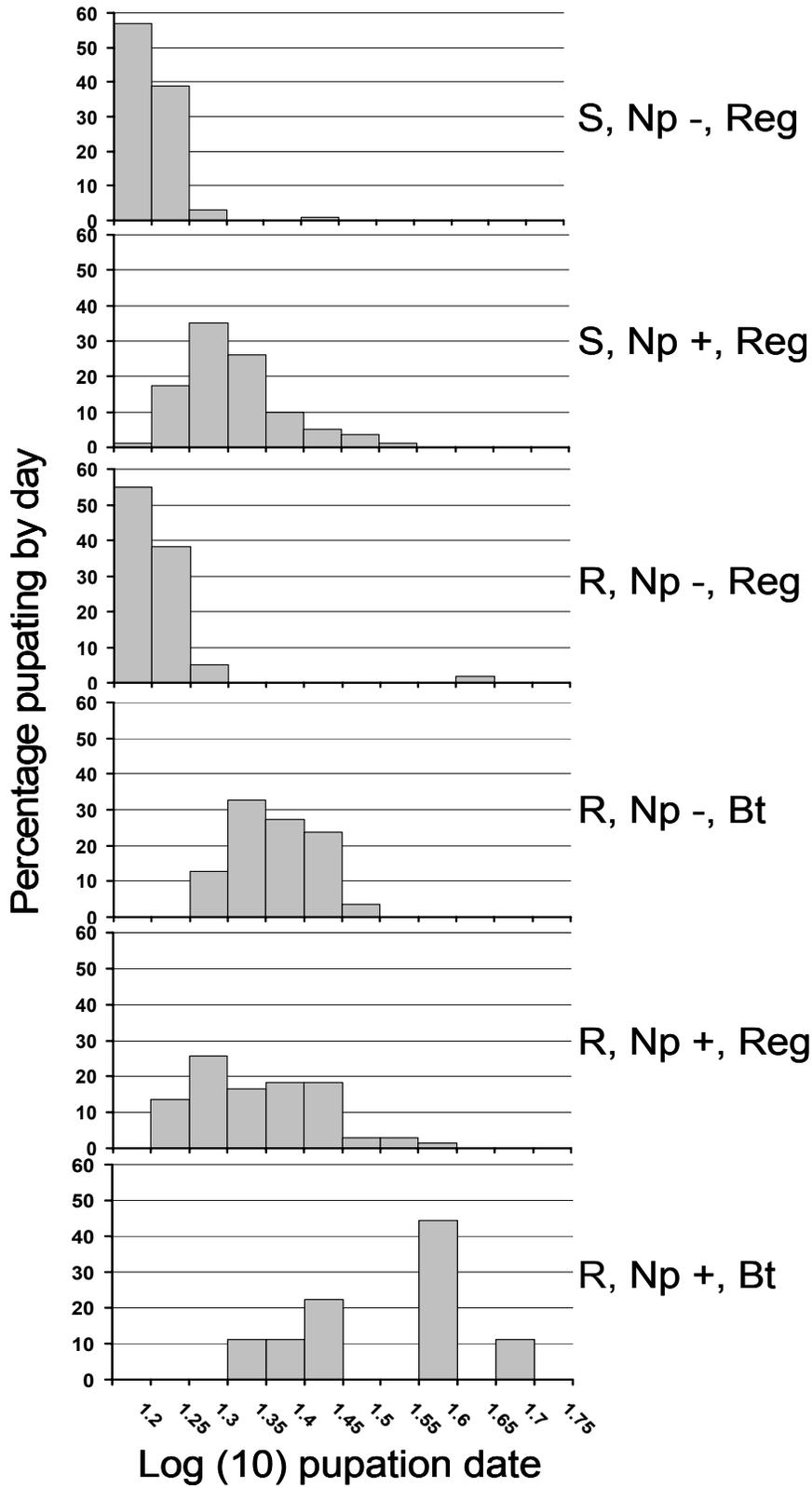


Fig. 6



## ACKNOWLEDGEMENTS

We thank J Dyer and R Gunnarson for technical assistance.

## REFERENCES

- Andreadis T. G. 1986.** Dissemination of *Nosema pyrausta* in feral populations of the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 48: 335-343.
- Andreadis T. G. 1987.** Horizontal transmission of *Nosema pyrausta* (Microsporida: Nosematidae) in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Environ. Entomol.* 16: 1124-1129.
- Bergman P. W., R. A. Davis, S. N. Fertig, F. Kuchler, R. McDowell, C. Osteen, A. L. Padula, K. L. Smith, and R. F. Torla. 1985.** Pesticide assessment of field corn and soybeans: corn belt states. *National Agricultural Pesticide Impact Assessment Program* (NAPIAP) Ext. Serv., U.S. Dept. Agric., Washington, DC.
- Bruck, D. J., M. D. Lopez, L. C. Lewis, J. R. Prasifka, and R. D. Gunnarson. 2007.** Effects of transgenic *Bacillus thuringiensis* corn and permethrin on nontarget arthropods. *J. Agric. Urban Entomol.* 23: 111-124.
- Caffery, D. L., and L. H. Worthley. 1927.** A progress report on the investigation of the European corn borer. *USDA Bull.* 1476.
- Dalecky, A., S. Ponsard, R. I. Bailey, C. Pélissier, and D Bourguet. 2006.** Resistance evolution to *Bt* crops: predispersal mating of European corn borers. *PLoS Biol.* 4(6): e181. DOI: 10.1371/journal.pbio.0040181.
- Fadamiro, H. Y., and T. C. Baker. 1999.** Reproductive performance and longevity of female European corn borer, *Ostrinia nubilalis*: effects of multiple mating, delay in mating, and adult feeding. *J. Insect Physiol.* 45: 385-392.
- Guthrie, W. D., E. S. Raun, F. F. Dicke, G. R. Pesho, and S. W. Carter. 1965.** Laboratory production of European corn borer egg masses. *Iowa State J. Sci.* 40: 65-83.
- Guthrie, W. D., J. C. Robbins, and J. L. Jarvis. 1985.** *Ostrinia nubilalis*, pp.407-413. In P. Singh & R.F. Moore [eds.], *Handbook of insect rearing*, vol. 2. Elsevier, New York.
- Inglis, G. D., A. M. Lawrence, and P. P. Sikorowski. 2003.** The use of meconia to nondestructively detect sublethal infections in Heliothines (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 96: 272-279.

- Lewis, L. C., and R. E. Lynch. 1970.** Treatment of *Ostrinia nubilalis* larvae with Fumidil B to control infections caused by *Perezia pyraustae*. *J. Invertebr. Pathol.* 15: 43-48.
- Liu, Y. B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, and A. C. Bartlett. 1999.** Development time and resistance to Bt crops. *Nature (Lond.)* 400:519.
- Liu, Y. B., B. E. Tabashnik, T. J. Dennehy, A. L. Patin, M. A. Sims, S. K. Meyer, and Y. Carrière. 2001.** Effects of Bt cotton and Cry1Ac toxin on survival and development of pink bollworm (Lepidoptera: Gelechiidae). *J. Econ. Entomol.* 94: 1237-1242.
- Mason, C. E., M. E. Rice, D. D. Calvin, J. W. Van Duyn, W. B. Showers, W. D. Hutchison, J. F. Witkowski, R. A. Higgins, D. W. Onstad, and G. P. Dively. 1996.** European corn borer ecology and management. North Central Regional Extension Pub. 327.
- Onstad D. W., and J. V. Maddox. 1989.** Modeling the effects of the microsporidium, *Nosema pyrausta*, on the population dynamics of the insect, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 53:410-421.
- Pesho, G. R. 1961.** Female mating patterns and spermatophore counts in the European corn borer, *Pyrausta nubilalis* (Hbn.). *Proceedings North Central Branch-ESA.* 16:43.
- Pierce, C. M. F., L. F. Solter, and R. A. Weinzierl. 2001.** Interactions between *Nosema pyrausta* (Microsporidia: Nosematidae) and *Bacillus thuringiensis* subsp. *kurstaki* in the European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 94: 1361-1368.
- Raymond, B., A. H. Sayyed, R. S. Hails, and D. J. Wright. 2007.** Exploiting pathogens and their impact on fitness costs to manage the evolution of resistance to *Bacillus thuringiensis*. *J. Appl. Ecol.* 44: 768-780.
- Reardon, B. J., R. L. Hellmich, D. V. Sumerford, and L. C. Lewis. 2004.** Growth, development, and survival of *Nosema pyrausta*-infected European corn borers (Lepidoptera: Crambidae) reared on meridic diet and Cry1Ab. *J. Econ. Entomol.* 97:1198-1201.
- Royer, L., and J. N. McNeil. 1993.** Male investment in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae): impact on female longevity and reproductive performance. *Functional Ecology* 7: 209-215.

- Sajap A. S., and L. C. Lewis. 1988.** Histopathology of transovarial transmission of *Nosema pyrausta* in the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 52: 147-153.
- Sajap A. S. and L. C. Lewis. 1992.** Chronology of infection of European corn borer (Lepidoptera: Pyralidae) with the microsporidium *Nosema pyrausta*: effect on development and vertical transmission. *Environ. Entomol.* 21: 178-182.
- Siegel J. P., J. V. Maddox, and W. G. Ruesink. 1986.** Lethal and sublethal effects of *Nosema pyrausta* on the European corn borer (*Ostrinia nubilalis*) in central Illinois. *J. Invertebr. Pathol.* 48: 167-173.
- Solter, L. F., J. V. Maddox, and D. W. Onstad. 1990a.** Transmission of *Nosema pyrausta* in adult European corn borers. *J. Invertebr. Pathol.* 57: 220-226.
- Solter, L. F., D. W. Onstad, and J. V. Maddox. 1990b.** Timing of disease-influenced processes in the life cycle of *Ostrinia nubilalis* infected with *Nosema pyrausta*. *J. Invertebr. Pathol.* 55: 337-341.
- Sumerford, D. V., L. C. Lewis, and J. C. Robbins. 2008.** Inheritance of resistance to Cry1Ab in a laboratory-selected colony of *Ostrinia nubilalis*: patterns of larval survival and larval development. Accepted to *Heredity*.
- United States Environmental Protection Agency (US EPA). 2001.** Biopesticides registration action document – *Bacillus thuringiensis* plant-incorporated protectants. 16 October 2001. Content accessed 30 October 2007. [http://www.epa.gov/pesticides/biopesticides/pips/bt\\_brad.htm](http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm).
- Wilson, R. L., and K. M. Wissink. 1986.** Laboratory method for screening corn for European corn borer (Lepidoptera: Pyralidae) resistance. *J. Econ. Entomol.* 79: 274-276.
- Windels M. B., H. C. Chiang, and B. Furgala. 1976.** Effects of *Nosema pyrausta* on pupa and adult stages of the European corn borer *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 27: 239-242.
- Zimmack, H.L., and T. A. Brindley. 1957.** The effect of the protozoan parasite *Perezia pyraustae* Paillot on the European corn borer. *J. Econ. Entomol.* 50: 637-640.

CHAPTER 3. PROCLIVITY OF TRANSMISSION OF *NOSEMA*  
*PYRAUSTA* FROM MALE EUROPEAN CORN BORERS TO THEIR  
OFFSPRING

A Scientific Note to be submitted to J. Invertebr. Pathol.

Miriam D. Lopez<sup>1,2</sup>, Leslie C. Lewis<sup>1</sup>

**ABSTRACT**

*Nosema pyrausta* Paillot (Microsporida: Nosematidae) is a pathogen that reduces mortality, fertility, and fecundity of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Infected females are known to transmit *N. pyrausta* transovarially to offspring, but ability of infected males to do the same is uncertain. Infected males were mated with non-infected females, and offspring examined for infection with *N. pyrausta*. No evidence of male transmission of *N. pyrausta* to offspring was found; male *O. nubilalis* either cannot vertically transmit *N. pyrausta*, or do so with such infrequency as to make it biologically insignificant.

**INTRODUCTION**

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is a serious pest of corn, and causes damage by tunneling within the corn stalk as well as by feeding on leaves and ears. Control methods that have been used for *O. nubilalis* include insecticides, host plant resistance, biological control, and transgenically modified plants. One organism that has been identified as being effective in suppressing populations of *O. nubilalis* is the microsporidium *Nosema pyrausta* Paillot (Microsporida: Nosematidae). *Nosema pyrausta* is an obligate parasite of *O. nubilalis* (Sajap and Lewis, 1988), and infection with *N. pyrausta* leads to decreased fecundity and survival (Zimmack and Brindley, 1957; Windels et al., 1976). While *N. pyrausta* will not completely protect crops from *O. nubilalis* damage, the sustained chronic effects of infection can reduce populations and may be able to negatively affect *O. nubilalis* levels

---

<sup>1</sup> Graduate student, Adjunct Professor, respectively, Department of Entomology, Iowa State University. USDA-ARS, Ames, Iowa.

<sup>2</sup> Primary researcher and author

(Onstad and Maddox, 1989). *Nosema pyrausta* can be transmitted vertically from female to offspring (Sajap and Lewis, 1988; 1992), or horizontally, by larvae feeding on spores deposited in frass (Andreadis, 1986; 1987). A study by Zimmack and Brindley (1957) examined mating of infected males to non-infected females, and concluded that male *O. nubilalis* do not vertically transmit *N. pyrausta* to offspring. However, another study seemingly demonstrated that infected males can transmit the microsporidium to offspring when mated with uninfected females, but reported that this occurrence was improbable (Solter et al., 1991). Larvae that become infected with *N. pyrausta* as early instars have increased negative effects of infection compared with larvae that encounter spores later in development (Sajap and Lewis, 1992). Vertical transmission of spores results in larvae that become infected during embryogenesis or at the time of eclosure (Kramer, 1959). Because *N. pyrausta* can act as a population regulator, efforts to increase the percentage of *O. nubilalis* infected with *N. pyrausta* may result in lower *O. nubilalis* populations and therefore reduced crop damage. Understanding the dynamics of transmission of *N. pyrausta* is important when considering interactions of the microsporidium in an integrated pest management strategy. The objective of this study was to determine whether a *N. pyrausta*-infected male could pass the infection to offspring when mated to a non-infected female. The results of this experiment will determine how insects are selected for paired matings to be used in experiments designed to determine how infection with *N. pyrausta* affects larvae resistant to Cry1Ab.

## MATERIALS AND METHODS

**Insect Preparation.** *Nosema pyrausta* infected population: A population of *O. nubilalis* infected with *N. pyrausta* was reared on meridic diet (Guthrie et al., 1985) containing 250 ppm Fumidil B, which maintains infection at a low level. Fumidil B is a protozoan static product that does not completely remove the microsporidium, but prevents it from completing the vegetative cycle and producing spores (Lewis and Lynch, 1970). To establish a generation of *O. nubilalis* with a greater infection level, eggs from this population were reared in a single container (25 cm diam., 9 cm deep) (Pioneer plastics, Dixon, KY) containing 1 L of meridic diet without Fumidil B. Because *O. nubilalis* larvae obtain infection with *N. pyrausta* by consuming frass containing viable

spores, communal rearing in an arena without Fumidil B facilitates spread of *N. pyrausta* through horizontal transmission to all larvae within the container. This rearing method resulted in virtually 100% infection of the larvae. Male pupae from this rearing method were used in experimental paired matings.

**Non-infected population:** A generation of *O. nubilalis* was reared using methods designed to prevent infection with *N. pyrausta* by eliminating horizontal transmission. Larvae from a non-infected population were reared in individual cells of meridic diet in condos sealed with porous covers (Oliver Medical, Grand Rapids, MI). This population was mostly free of infection with *N. pyrausta*, but isolated instances of infection were found. Because of this, any individual used from this population was later checked for *N. pyrausta* to confirm infection status. Fumidil B was not included in the diet thus if an infection was present it would be detectable. Female pupae from this population were used in experimental paired matings.

**Paired matings.** Single pair mating cages (Kira et al., 1969) were established with one infected male and one uninfected female. To determine infection status of offspring, eggs were collected from cages on a daily basis. One-two fertile egg masses from each mated pair each day were placed into 18 ml plastic cups (Anderson Tool and Die, Linden, NJ) of diet without Fumidil B, covered with unwaxed lids (Stanpac, Lewiston, NY) and allowed to hatch. Communal rearing in the absence of Fumidil B allows horizontal transmission of *N. pyrausta*, and takes advantage of crowding interactions to magnify any possible infection, maximizing the potential of finding any infected offspring resulting from mating between an infected male and a non-infected female. After 10 days at optimum larval development conditions, 10 larvae from each cup were evaluated for infection (+ or -) as follows: 3<sup>rd</sup> to 4<sup>th</sup> instar larvae were placed into individual tissue grinders and approximately 1 ml of water was added. Entire larvae were homogenized and a small amount of the resulting liquid was placed onto a slide and examined using a light microscope (400X). Because the spore count of *N. pyrausta* in offspring increases over time as well as the resulting mortality (Sajap and Lewis, 1992; Siegel et al., 1986; Bruck et al., 2001), only eggs from the last oviposition date were evaluated. At death of female or after completion of egg laying, mated pairs were frozen

for later evaluation to confirm infection status. For each replicate 20 single pair mating cages were established, and any pairs that produced fertile eggs were used for evaluation of male vertical (venereal) transmission.

## RESULTS

A total of 25 mated pairs from the 3 replicates produced fertile egg masses. After eclosion, at least 10 larvae were examined from each mated pair. There was no evidence of *N. pyrausta* in the offspring of twenty mated pairs (Table 1). There were 5 mated pairs that had offspring positive for *N. pyrausta*, 2 in Rep 2 and 3 in Rep 3, however careful postmortem dissection of the presumed infection-free females from these 5 pairs revealed infection with *N. pyrausta*, which ostensibly led to the infected offspring. Females were meticulously dissected beginning at the anterior portion of the abdomen, avoiding the posterior bursa copulatrix containing the spermatophore. The midgut and malpighian tubule tissues were analyzed and found to be highly infected in all cases. Previous dissections of the bursa copulatrix from non-infected females revealed no evidence of *N. pyrausta* spores on or in spermatophores deposited by infected males (unpublished data). If, however, venereal transmission of *N. pyrausta* from male to female via transfer of spores on or in the spermatophore is possible, it was not shown in this experiment. The level of infection in the females for this research was very high, thus it would not have been possible for a venereal contamination to reach this high level of infection during the time span between mating and female sacrifice or death. The twenty pairs comprised of infected males and (correctly) non-infected females showed no evidence of male vertical transmission of *N. pyrausta* to offspring.

**Table 1: Total number of successful paired matings between a *N. pyrausta*-infected male and a presumed non-infected female, number of paired matings which resulted in all negative offspring, and number of paired matings that resulted in some offspring positive for *N. pyrausta*.**

<i>Replication</i>	<i>Total number of paired matings</i>	<i>All offspring negative</i>	<i>Some positive offspring</i>
1	8	8	0
2	10	8	2*
3	7	4	3*

\* Postmortem examination confirmed that females were positive for *N. pyrausta*

## DISCUSSION

There was no evidence of male vertical transmission of *N. pyrausta* to offspring. These findings are important to our understanding of the dynamics of *N. pyrausta*, especially the mechanics of transmission in the environment, and will help us develop concepts to conserve this naturally-occurring biological control agent. Other published work reports the possibility of venereal transmission of *N. pyrausta* by infected males (Solter et al., 1991). In that study there were no controls and there were no indications that there was a post mortem evaluation to verify the infectivity state of the test insects. Solter et al. (1991) alluded to the fact that if venereal transmission does occur it is most likely of little consequence in nature. Under the conditions that this research was conducted there is no evidence that there is venereal transmission of *N. pyrausta*. Within this body of experimentation, knowing that male *O. nubilalis* do not contribute to venereal transmission of *N. pyrausta* in the field provides a framework for answering questions that call for vertically infected offspring.

## REFERENCES

- Andreadis T. G. 1986.** Dissemination of *Nosema pyrausta* in feral populations of the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 48: 335-343.
- Andreadis T. G. 1987.** Horizontal transmission of *Nosema pyrausta* (Microsporida: Nosematidae) in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Environ. Entomol.* 16: 1124-1129.
- Bruck, D. J., L. C. Lewis, and R. D. Gunnarson. 2001.** Interaction of *Nosema pyrausta* and temperature on *Ostrinia nubilalis* egg production and hatch. *J. Invertebr. Pathol.* 78: 210-214.
- Guthrie, W. D., J. C. Robbins, and J. L. Jarvis. 1985.** *Ostrinia nubilalis*, pp.407-413. *In* P. Singh & R.F. Moore [eds.], *Handbook of insect rearing*, vol. 2. Elsevier, New York.
- Kira, M. T., W. D. Guthrie, and J. L. Huggans. 1969.** The effect of drinking water on production of eggs by the European corn borer. *Iowa State J. Sci.* 41: 963-970.
- Kramer, J. P. 1959.** Some relationships between *Perezia pyraustae* Paillot (Sporozoa, Nosematidae) and *Pyrausta nubilalis* (Hübner) (Lepidoptera: Pyralidae). *J. Insect Pathol.* 1: 25-33.

- Lewis, L.C., and R. E. Lynch. 1970.** Treatment of *Ostrinia nubilalis* larvae with Fumidil B to control infections caused by *Perezia pyraustae*. J. Invertebr. Pathol. 15: 43-48.
- Onstad D. W., and J. V. Maddox. 1989.** Modeling the effects of the microsporidium, *Nosema pyrausta*, on the population dynamics of the insect, *Ostrinia nubilalis*. J. Invertebr. Pathol. 53: 410-421.
- Sajap A. S., and L. C. Lewis. 1988.** Histopathology of transovarial transmission of *Nosema pyrausta* in the European corn borer, *Ostrinia nubilalis*. J. Invertebr. Pathol. 52: 147-153.
- Sajap A. S., and L. C. Lewis. 1992.** Chronology of infection of European corn borer (Lepidoptera: Pyralidae) with the microsporidium *Nosema pyrausta*: effect on development and vertical transmission. Environ. Entomol. 21: 178-182.
- Siegel J. P., J. V. Maddox, and W. G. Ruesink. 1986.** Lethal and sublethal effects of *Nosema pyrausta* on the European corn borer (*Ostrinia nubilalis*) in central Illinois. J. Invertebr. Pathol. 48: 167-173.
- Solter L. F., J. V. Maddox, and D. W. Onstad. 1991.** Transmission of *Nosema pyrausta* in adult European corn borers. J. Invertebr. Pathol. 57: 220-226.
- Windels M. B., H. C. Chiang, and B. Furgala. 1976.** Effects of *Nosema pyrausta* on pupa and adult stages of the European corn borer *Ostrinia nubilalis*. J. Invertebr. Pathol. 27: 239-242.
- Zimmack H. L., and T. A. Brindley. 1957.** The effect of the protozoan parasite *Perezia pyraustae* Paillot on the European corn borer. J. Econ. Entomol. 50: 637-640.

CHAPTER 4. EFFECTS OF INFECTION WITH *NOSEMA PYRAUSTA*  
ON SURVIVAL AND DEVELOPMENT OF OFFSPRING OF  
LABORATORY SELECTED BT-RESISTANT AND BT-SUSCEPTIBLE  
EUROPEAN CORN BORERS

A paper to be submitted to J. Econ. Entomol.

Miriam D. Lopez<sup>1,2</sup>, Douglas V. Sumerford<sup>1</sup>, Leslie C. Lewis<sup>1</sup>

**ABSTRACT**

Infection with *Nosema pyrausta* Paillot (Microsporida: Nosematidae) lengthens developmental period of Bt-susceptible *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) to a similar extent as feeding on Cry1Ab-incorporated diet in Cry1Ab-resistant *O. nubilalis*, and these two factors combined lengthen developmental period further than either alone. These developmental delays may increase probability of mating between non-infected resistant *O. nubilalis* and *N. pyrausta*-infected susceptible *O. nubilalis*, as well as probability of assortative mating within *N. pyrausta*-infected resistant *O. nubilalis* populations. Test crosses produced partially- and fully Cry1Ab-resistant *O. nubilalis* infected and not-infected with *N. pyrausta*, which were exposed to Cry1Ab toxin at doses of 0, 3, or 30 ng/cm<sup>2</sup> for 7 days. Infection with *N. pyrausta* significantly decreased 7 day survival of partially and fully-resistant *O. nubilalis* on 30 ng/cm<sup>2</sup> Cry1Ab. *Nosema pyrausta* infection delayed larval development (as measured by weight) of partially- and fully-resistant *O. nubilalis* on 3 and 30 ng/cm<sup>2</sup> Cry1Ab. Nonrandom mating of *O. nubilalis* due to differential developmental rates may increase the likelihood of resistant adults mating with infected susceptible, or infected resistant partners. This would produce partially- and fully-resistant offspring, respectively, infected with *N. pyrausta*. *Nosema pyrausta*-infected *O. nubilalis* are more strongly affected by feeding on Bt, and would be less likely to survive to adulthood. This

---

<sup>1</sup> Graduate student, Adjunct Assistant Professor, Adjunct Professor, respectively, Department of Entomology, Iowa State University. USDA-ARS, Ames, Iowa.

<sup>2</sup> Primary researcher and author

indigenous microsporidium may work to delay evolution of resistance in *O. nubilalis* by lowering their ability to survive on Bt.

## INTRODUCTION

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) is a serious pest of corn that inflicts injury resulting in yield loss (Mason et al., 1996). To prevent yield loss due to *O. nubilalis*, scientists have inserted genetic material from a bacterium, *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) into the genome of corn. This genetically-modified (Bt) corn produces a toxin in its tissues that is lethal to *O. nubilalis* when ingested. A major concern regarding the effective lifespan of Bt corn technology is the possibility of resistance development. Currently, to combat resistance development, a requirement for planting Bt corn is the high dose/refuge strategy (U.S. EPA, 2001). One condition of this strategy is that the dose of toxin expressed within the plant be a high dose to eliminate 99.9% of individuals heterozygous for a resistance allele (“functionally recessive”), and assumes that such alleles are rare in the population. The corresponding requirement is that a specified percentage (currently 20% in the corn belt) of corn be planted to non-Bt corn as a refuge. The defined purpose of the refuge is to produce sufficient numbers of genetically-susceptible adults to mate with resistant adults to reduce the frequency of resistance alleles, but it may also help control *O. nubilalis* by conserving natural enemies (Bruck et al., 2007). One assumption of the high dose/refuge strategy is that resistant adults emerging from Bt corn will mate randomly or preferentially with susceptible adults from the refuge. Mating populations of refuge and resistant adults would need to be present at the same time in order for random mating to occur. Because matings between heterozygous-resistant and susceptible adults will never produce homozygous-resistant offspring, this strategy should prevent evolution of resistance to Bt corn.

However, it was found in a recent study that laboratory selected Cry1Ab-resistant *O. nubilalis* larvae feeding on Cry1Ab-incorporated diet had significantly longer developmental periods compared with their susceptible and resistant counterparts on non-Bt diet (See Ch. 2). It was also found that infection with the microsporidium *Nosema pyrausta* Paillot (Microsporida: Nosematidae) lengthened developmental periods (See

Ch. 2), which mirrors other work (Solter et al., 1990b). *Nosema pyrausta* is a widespread obligate parasite of *O. nubilalis* that causes decreased fecundity and survival (Solter et al., 1990b; Sajap and Lewis 1992). If the developmental delays seen in laboratory experiments that used Cry1Ab toxin-incorporated diet emulate what would happen in a field situation, resistant *O. nubilalis* developing on Bt corn will emerge later than uninfected *O. nubilalis* from the refuge. Susceptible *O. nubilalis* infected with *N. pyrausta* will also experience delayed emergence relative to uninfected *O. nubilalis*. Because of the developmental delay, Bt-resistant moths may be more likely to encounter similarly-delayed, *N. pyrausta*-infected moths from the refuge than non-infected refuge moths, but this is dependant on percentage of *O. nubilalis* infected with *N. pyrausta*. Previous studies have shown increased detrimental effects to Bt-susceptible *O. nubilalis* infected with *N. pyrausta* when challenged with Bt compared with uninfected *O. nubilalis* (Reardon et al., 2004; Pierce et al., 2001; See Ch. 2). Little work has been done to elucidate how infection with *N. pyrausta* may influence evolution of resistance to Bt corn. If a Bt-resistant moth mates with a *N. pyrausta*-infected refuge moth and the infection is passed on transovarially, their heterozygous-resistant offspring may be less vigorous on Bt than the uninfected offspring resulting from a mating with a non-infected refuge moth. This phenomenon has the potential to influence the rate of resistance evolution.

This experiment was designed to investigate the effect of transovarial infection with *N. pyrausta* on the offspring resulting from both resistant-susceptible matings, and resistant-resistant matings. These offspring will be partially or fully resistant to Cry1Ab. The objective of this study was to determine the effects of exposure to two levels of Cry1Ab-toxin on larval development and mortality of 4 populations of *O. nubilalis*: 1) resistant individuals, transovarially infected with *N. pyrausta*, 2) resistant individuals, non-infected, 3) individuals receiving 50% of their genetic background from the resistant population, transovarially infected with *N. pyrausta*, and 4) individuals receiving 50% of their genetic background from the resistant population, non-infected.

## MATERIALS AND METHODS

**Insect Preparation.** Four populations of *O. nubilalis* were established in the laboratory and later bred to produce offspring having the desired resistance and infection types: 1) resistant, non-infected, 2) resistant, *N. pyrausta*-infected, 3) susceptible, non-infected, and 4) susceptible, *N. pyrausta*-infected. A population of *O. nubilalis* resistant (“RES”) to high levels of the Bt protein Cry1Ab has been selected for and maintained in the laboratory for >50 generations (Sumerford et al., 2008). This population is greater than 2500x resistant and will feed and survive on reproductive stage corn. Eggs from the RES population were used to create infected and non-infected populations as follows: half of the eggs were placed into rearing containers (25 cm diameter) (Pioneer Plastics, Inc., Dixon, KY) containing 930 g meridic diet (Guthrie et al., 1985), which were inoculated with *N. pyrausta* (1000 spores/mm<sup>2</sup> diet) at 3-4 days post egg hatch to establish the resistant infected population. The remaining eggs were placed into containers of meridic diet without spores to establish the non-infected population. A population free from infection with *N. pyrausta* is continuously maintained in the laboratory, and eggs from this population were used to establish infected and non-infected populations as described above. These four populations were mated to produce transovarially infected and non-infected F<sub>1</sub> offspring. Test populations resulting from mass matings using the four populations described above were established to produce larvae partially - (F<sub>1</sub> populations #1 and #2, Table 1, 50% of their genetic material from a susceptible female) or fully-resistant to Cry1Ab (#3 and #4, Table 1). Sumerford et al. (2008) has shown that the resistance selected for is not sex-linked. Reciprocal crosses using resistant females crossed to susceptible males would have been redundant with respect to the resistance phenotype.

*N. pyrausta* can be transmitted to the offspring of an infected female via transovarial transmission (Sajap and Lewis, 1988; 1992). Male *O. nubilalis* are either incapable of transmitting *N. pyrausta* infection to offspring, or, if it does occur, is very infrequent and unlikely to contribute to spread of the disease in the field (Solter et al., 1990a; Ch. 3). Therefore, in matings requiring an infected F<sub>1</sub> population, the female was always the infected partner. Before placement into mating arenas, infection status for all

emerged females was verified by examining the meconium for *N. pyrausta* spores (Inglis et al., 2003). All females that came from inoculated containers were positive, and all that came from control containers were negative. Between 20 and 30 adults of each sex were placed into mating arenas in the combinations shown in Table 1. Eggs were collected daily and maintained at 27°C, 70% relative humidity (Rh), and continuous light until hatch.

**Table 1: Test Crosses**

<i>Female</i>	<i>Male</i>	<i>F<sub>1</sub> Offspring</i>
Susceptible, uninfected	Resistant, non-infected	1) Partially-resistant, uninfected
Susceptible, <i>N. pyrausta</i>	Resistant, non-infected	2) Partially-resistant, <i>N. pyrausta</i>
Resistant, uninfected	Resistant, non-infected	3) Resistant, uninfected
Resistant, <i>N. pyrausta</i>	Resistant, non-infected	4) Resistant, <i>N. pyrausta</i>

**Diet and experimental arenas.** The surface overlay method developed by Siegfried et al. (2001) was used to bioassay Cry1Ab resistance. Meridic diet was prepared, metered into opaque 128 cell Bio-Assay trays (Bio-serv, Frenchtown, NJ) using a repetitive pipetter, 1 ml per cell, taking great care to prevent any surface imperfections such as bubbles or depressions, and allowed to solidify overnight. Cry1Ab toxin (trypsin-activated Cry1Ab; obtained from Dr. M. Carey, Dept. of Biochemistry, Case Western Reserve University, Cleveland, OH), was mixed in 0.1% Triton-X 100 non-ionic detergent (used to ensure even spread across the diet surface), and pipetted onto the diet surface. Larvae were exposed to two doses of Cry1Ab; a low, sublethal dose (3 ng/cm<sup>2</sup>) and a high dose (30 ng/cm<sup>2</sup>). The current diagnostic dose of Cry1Ab used to monitor resistance in field populations of *O. nubilalis* is 10 ng/cm<sup>2</sup> (Marçon et al., 2000). These doses along with a control of 0.1% Triton-X 100 were overlaid onto the diet cells using a repetitive pipetter (30 µl), and allowed to dry (see Siegfried et al., 2001). Neonatal *O. nubilalis* were placed one per cell onto the control diet or diet overlaid with one of two doses of Cry1Ab toxin. Trays were sealed with 16-cell self-adhesive Bio-Assay tray lids (Bio-serv) that allowed for air exchange and prevented larval movement between cells.

Trays were maintained at ideal larval conditions (27°C, 70% Rh, and continuous light) for 7 days. Data were recorded from all larvae at 7 days, and included weight and survival.

**Experimental design and data analysis.** The experiment was designed as a split-split plot with four replicates. Each 128-cell tray was considered a replicate with diet considered as one strip and F<sub>1</sub> populations as the other strip. For each replicate, 8, 12, and 12 neonates per F<sub>1</sub> population were exposed to control, “low”, and “high” doses of Cry1Ab, respectively. For analysis purposes, any larva that did not molt (<0.3 g at 7 days) was considered dead. Weight data were Log<sub>10</sub>-transformed to enhance normality and produce comparable variances among treatments.

All data were modeled using restricted-maximum-likelihood estimates for the mixed-model analysis of variance. Proc Mixed of SAS (v. 9.1.3) was used to perform the analysis. Diet, F<sub>1</sub> population and their interactions were considered fixed effects. Random sources of variance included replicate effects and their interactions with fixed effects.

## RESULTS

**Larval survival.** The three-way interaction of resistance level, *N. pyrausta* infection, and diet was significant (Table 2). Infection with *N. pyrausta* did not affect fully-resistant or partially-resistant larval survival on control or low dose Cry1Ab diet (Fig. 1). Additionally, low and high doses of Cry1Ab toxin did not significantly decrease survival of uninfected RR or RS larvae. However, when *N. pyrausta*-infected RR and RS larvae were exposed to high dose Cry1Ab, survival was significantly decreased compared to uninfected larvae.

**Larval weight.** Feeding on high dose Cry1Ab negatively impacted development of RR larvae as measured by weight, and both low and high doses of Cry1Ab negatively impacted development of RS larvae compared with control diet (Fig. 2). Resistance level (full or partial) also influenced development of uninfected larvae on low and high doses of Cry1Ab, with RS weights significantly lower than RR weights at 7 days. Infection with *N. pyrausta* significantly slowed development in both RR and RS populations on both Cry1Ab diets relative to uninfected (Fig. 2). The combination of infection with *N. pyrausta* and feeding on Bt diet led to lower weights in both RR and RS larvae than

either factor alone. The three-way interaction of resistance level, *N. pyrausta* infection, and diet was not significant (Table 3), because the effect of *N. pyrausta* was the same on all diets.

**Table 2: Larval survival test of fixed effects**

<i>Effect</i>	<i>Numerator DF</i>	<i>Denominator DF</i>	<i>F</i>	<i>P</i>
Resistance Status	1	9	8.87	0.0155
Disease Status	1	9	58.34	<.0001
Diet	2	6	58.54	0.0001
Resistance x Disease	1	9	0.82	0.3884
Resistance x Diet	2	18	14.30	0.0002
Disease x Diet	2	18	33.70	<.0001
Resistance x Disease x Diet	2	18	8.16	0.0030

**Table 3: Larval weight, test of fixed effects**

<i>Effect</i>	<i>Numerator DF</i>	<i>Denominator DF</i>	<i>F</i>	<i>P</i>
Resistance Status	1	9	46.45	<.0001
Disease Status	1	9	452.11	<.0001
Resistance x Disease	1	9	34.78	0.0002
Diet	2	6	177.88	<.0001
Resistance x Diet	2	18	51.48	<.0001
Disease x Diet	2	18	4.55	0.0252
Resistance x Disease x Diet	2	18	1.72	0.2079

## DISCUSSION

If resistant *O. nubilalis* experience delayed emergence due to feeding on Bt corn, they may be more likely to encounter *N. pyrausta*-infected moths from the refuge than non-infected moths, because infected larvae also exhibit delayed development (See Ch. 2). When a fully resistant male mates with a *N. pyrausta*-infected susceptible female, all of their offspring will be partially resistant, and many will be infected with *N. pyrausta*. The percentage of offspring that will be infected is dependent on when the larval female

encountered *N. pyrausta* spores, the number of spores ingested, and day of oviposition, with later egg dates exhibiting higher percentage infection (Sajap and Lewis, 1992). The offspring from matings designed to simulate this phenomenon (partially-resistant, *N. pyrausta*-infected) were less vigorous overall compared to their uninfected counterpart, and when challenged with a high dose of Cry1Ab toxin, had dramatically lower survival and slower development. In this scenario, partially-resistant *O. nubilalis* that were infected with *N. pyrausta* as a result of mating between resistant moths and susceptible, infected moths would be unlikely to survive on Bt to contribute their genetic material to the following generation, slowing the development of resistance. Because infected larvae experience greater mortality on Bt, *N. pyrausta* may represent an avenue by which resistant *O. nubilalis* can be made more susceptible to Bt, and therefore reduce survival and subsequent transmission of resistance alleles.

Assortative mating of resistant moths due to their delayed emergence relative to refuge moths would lead to faster resistance evolution regardless of refuge size. Infection with *N. pyrausta* could further delay emergence in resistant *O. nubilalis* feeding on Bt (See Ch. 2). Because *N. pyrausta*-infected resistant larvae fed on Cry1Ab-incorporated diet are severely delayed, there may be assortative mating within this group. In this scenario, assortative mating of infected resistant moths would result in fully-resistant offspring, some infected with *N. pyrausta*. The experimental treatments designed to simulate this scenario exposed offspring that were homozygous, uninfected, and homozygous, *N. pyrausta*-infected to non-Bt diet as well as low and high doses of Cry1Ab. The *N. pyrausta*-infected larvae were overall less vigorous than the uninfected as measured by weight at 7 days (Fig. 2). When exposed to low and high doses of Cry1Ab, the *N. pyrausta*-infected larvae had significantly slower development compared with uninfected larvae. Relative to the non-Bt treatment, survival was reduced on high dose Cry1Ab for fully-resistant, *N. pyrausta*-infected larvae, but was not significantly reduced at the low dose. Even though survival at 7 days was not significantly decreased for all infected/Cry1Ab treatments, the developmental delays caused by exposure to Cry1Ab (and evidenced by the low weights) could allow for increasing intensity of *N. pyrausta* infection over time, eventually leading to higher levels of mortality, and less

chance of transmitting resistance to subsequent generations. Bruck et al. (2001) performed an experiment showing that when *O. nubilalis* moths were held at cool temperatures for extended periods of time, *N. pyrausta* built up in their reproductive systems, leading to reduced fecundity. The same process of *N. pyrausta* build-up could be occurring in larvae feeding on Bt and could eventually lead to larval death. If overwintering larvae are infected with *N. pyrausta*, there is an even longer period of time for *N. pyrausta* intensity to potentially increase, which may influence the success of generation 1 the following year.

If genotypically resistant moths mate only with one another because the refuge moths have previously emerged and completed mating, the rate of response to selection for resistance to Bt will be drastically increased. Resistant adults mating with *N. pyrausta*-infected susceptible adults will produce genotypically partially-resistant offspring, but the combination of feeding on Bt plants and having a microsporidian infection may render the offspring phenotypically susceptible to Bt, thus removing their contribution to the next generation. The corn refuge, in addition to producing susceptible adults to reduce the frequency of resistance alleles, may also serve as a reservoir of *N. pyrausta*, which has the potential to increase the susceptibility of infected resistant individuals to Bt. Levels of *N. pyrausta* fluctuate year to year, and it is a density dependent pathogen (Siegel et al., 1988; Hill and Gary, 1979; Lewis et al., 2006). During years of high *O. nubilalis* populations there will be more potentially-resistant individuals present, however there may also be more *N. pyrausta*-infected individuals in the refuge. Resistant moths mating with these infected refuge moths will produce offspring more susceptible to Bt than they would if they mated with uninfected refuge moths. Efforts to conserve *N. pyrausta* in the corn ecosystem and facilitate transmission between insects may work to slow the evolution of resistance to Bt, ensuring the continued effectiveness of the technology.

#### FIGURE CAPTIONS

Figure 1. 7 day survival of fully-resistant (RR) and partially-resistant (RS) *O. nubilalis* larvae infected (Np +) and not infected (Np -) with *N. pyrausta* on control diet (Reg), 3 ng/cm<sup>2</sup> Cry1Ab-overlaid diet (Low) and 30 ng/cm<sup>2</sup> Cry1Ab-overlaid diet (High). Proc Mixed was used to generate

survival estimates. Estimates followed by the same letter are not significantly different.

Figure 2. Larval weight at 7 days of fully-resistant (RR) and partially-resistant (RS) *O. nubilalis* larvae infected (Np +) and not infected (Np -) with *N. pyrausta* on control diet (Reg), 3 ng/cm<sup>2</sup> Cry1Ab-overlaid diet (Low) and 30 ng/cm<sup>2</sup> Cry1Ab-overlaid diet (High). Proc Mixed was used to generate weight estimates. Estimates followed by the same letter are not significantly different.

Fig. 1

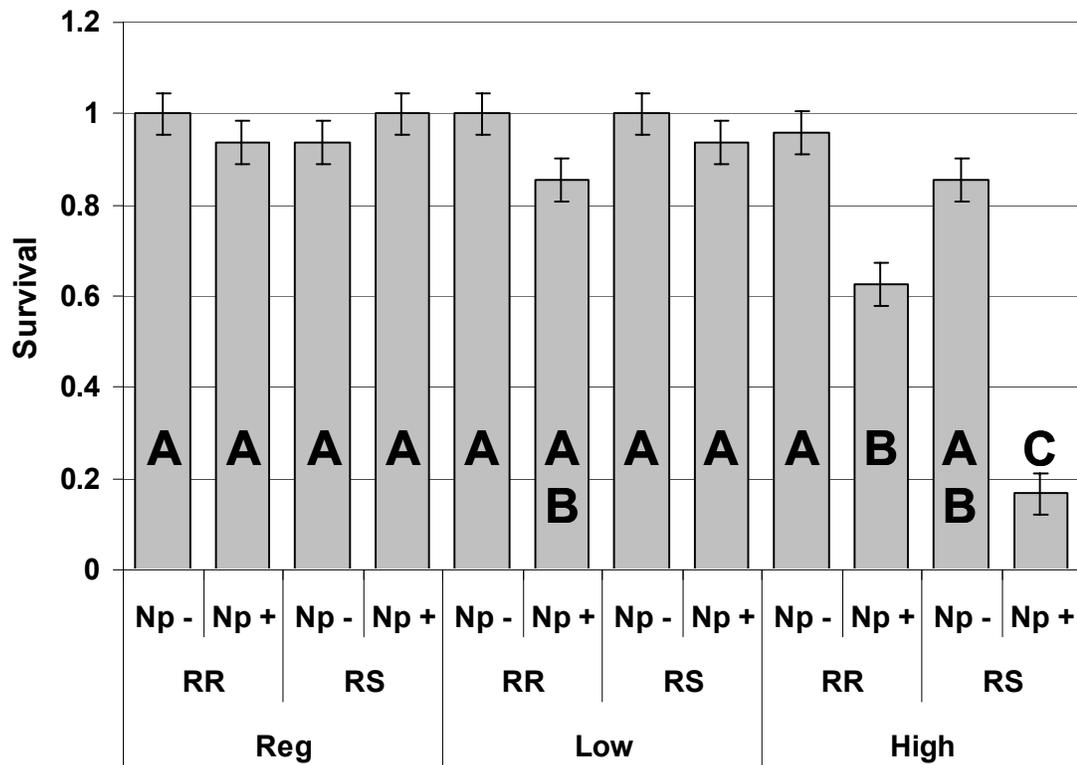
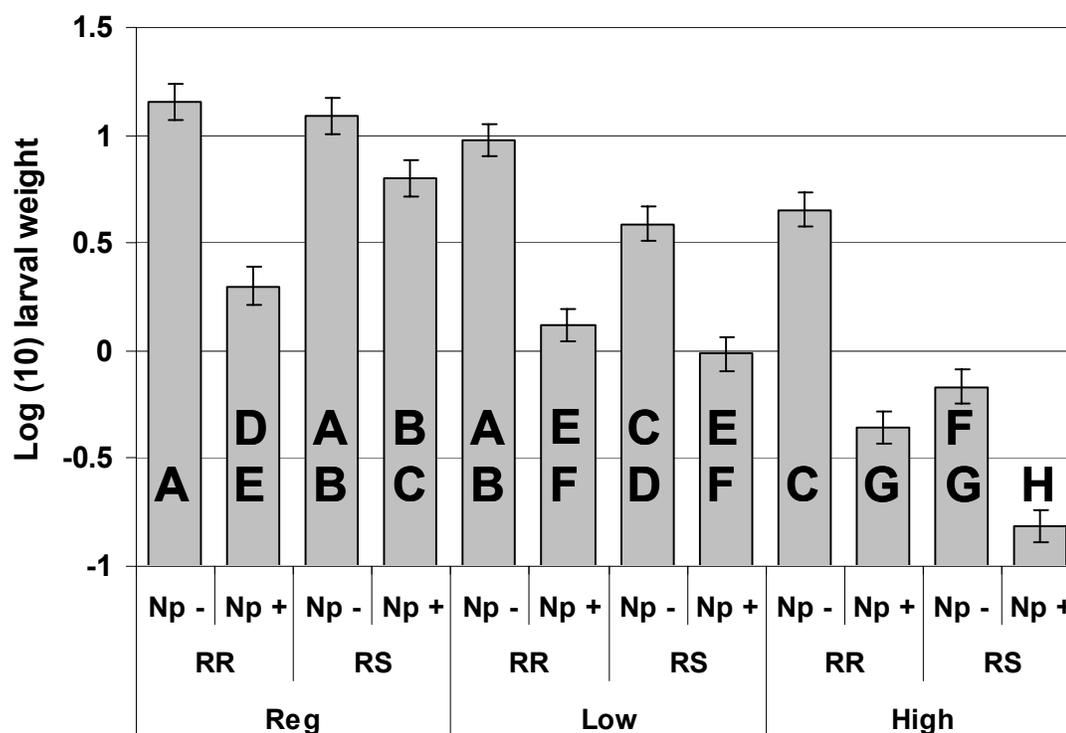


Fig. 2



#### ACKNOWLEDGEMENTS

We thank R Gunnarson, J Robbins, and D Steines for technical assistance.

#### REFERENCES

- Bruck, D. J., L. C. Lewis, and R. D. 2001.** Interaction of *Nosema pyrausta* and temperature on *Ostrinia nubilalis* egg production and Hatch. *J. Invertebr. Pathol.* 78: 210-214.
- Bruck, D. J., M. D. Lopez, L. C. Lewis, J. R. Prasifka, and R. D. Gunnarson. 2007.** Effects of transgenic *Bacillus thuringiensis* corn and permethrin on nontarget arthropods. *J. Agric. Urban Entomol.* 23: 111-124.
- Guthrie, W. D., J. C. Robbins, and J. L. Jarvis. 1985.** *Ostrinia nubilalis*, pp.407-413. In P. Singh & R.F. Moore [eds.], *Handbook of insect rearing*, vol. 2. Elsevier, New York.
- Hill, R. E., and W. J. Gary. 1979.** Effects of the Microsporidium, *Nosema pyrausta*, on field populations of European corn borers in Nebraska. *Environ. Entomol.* 8: 91-95.

- Inglis, G. D., A. M. Lawrence, and P. P. Sikorowski. 2003.** The use of meconia to nondestructively detect sublethal infections in Heliothines (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 96: 272-279.
- Lewis, L. C., D. V. Sumerford, L. A. Bing, and R. D. Gunnarson. 2006.** Dynamics of *Nosema pyrausta* in natural populations of the European corn borer, *Ostrinia nubilalis*: a six-year study. *BioControl* 51: 627-642.
- Marçon, P. C. R. G., B. D. Siegfried, T. Spencer, and W. D. Hutchison. 2000.** Development of diagnostic concentrations for monitoring *Bacillus thuringiensis* resistance in European corn borer (Lepidoptera: Crambidae). *J. Econ. Entomol.* 93: 925-930.
- Mason, C. E., M. E. Rice, D. D. Calvin, J. W. Van Duyn, W. B. Showers, W. D. Hutchison, J. F. Witkowski, R. A. Higgins, D. W. Onstad, and G. P. Dively. 1996.** European corn borer ecology and management. North Central Regional Extension Pub. 327.
- Pierce, C. M. F., L. F. Solter, and R. A. Weinzierl. 2001.** Interactions between *Nosema pyrausta* (Microsporidia: Nosematidae) and *Bacillus thuringiensis* subsp. *kurstaki* in the European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 94: 1361-1368.
- Reardon, B. J., R. L. Hellmich, D. V. Sumerford, and L. C. Lewis. 2004.** Growth, development, and survival of *Nosema pyrausta*-infected European corn borers (Lepidoptera: Crambidae) reared on meridic diet and Cry1Ab. *J. Econ. Entomol.* 97: 1198-1201.
- Sajap A. S., and L. C. Lewis. 1988.** Histopathology of transovarial transmission of *Nosema pyrausta* in the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 52: 147-153.
- Sajap A. S., and L. C. Lewis. 1992.** Chronology of infection of European corn borer (Lepidoptera: Pyralidae) with the microsporidium *Nosema pyrausta*: effect on development and vertical transmission. *Environ. Entomol.* 21: 178-182.
- SAS Institute. 2004.** (Version 9.1.3), SAS Institute, Cary, NC.
- Siegel, J. P., J. V. Maddox, and W. G. Ruesink. 1988.** Seasonal progress of *Nosema pyrausta* in the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 52: 130-136.
- Siegfried, B. D., A. C. Zoerb, and T. Spencer. 2001.** Development of European corn borer larvae on event 176 Bt corn: influence on survival and fitness. *Entomol. Exp. Appl.* 100: 15-20.

- Solter, L. F., J. V. Maddox, and D. W. Onstad. 1990a.** Transmission of *Nosema pyrausta* in adult European corn borers. *J. Invertebr. Pathol.* 57: 220-226.
- Solter, L. F., D. W. Onstad, and J. V. Maddox. 1990b.** Timing of disease-influenced processes in the life cycle of *Ostrinia nubilalis* infected with *Nosema pyrausta*. *J. Invertebr. Pathol.* 55: 337-341.
- Sumerford, D. V., L. C. Lewis, and J. C. Robbins. 2008.** Inheritance of resistance to Cry1Ab in a laboratory-selected colony of *Ostrinia nubilalis*: patterns of larval survival and larval development. Accepted to *Heredity*.
- United States Environmental Protection Agency (US EPA). 2001.** Biopesticides registration action document – *Bacillus thuringiensis* plant-incorporated protectants. 16 October 2001. Content accessed 30 October 2007. [http://www.epa.gov/pesticides/biopesticides/pips/bt\\_brad.htm](http://www.epa.gov/pesticides/biopesticides/pips/bt_brad.htm).

CHAPTER 5. ENCOUNTERING *NOSEMA PYRAUSTA* AND BT:  
EFFECTS ON LATE-INSTAR CRY1AB-RESISTANT AND  
SUSCEPTIBLE EUROPEAN CORN BORERS

A paper to be submitted to *Entomologia experimentalis et applicata*

Miriam D. Lopez<sup>1,2</sup>, Douglas V. Sumerford<sup>1</sup>, Leslie C. Lewis<sup>1</sup>

**ABSTRACT**

*Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) may deposit spores of, and become infected by *Nosema pyrausta* Paillot (Microsporida: Nosematidae) via horizontal transmission. *Nosema pyrausta* has been shown to increase mortality of vertically-infected Cry1Ab-resistant neonatal larvae on Cry1Ab-incorporated diet. This experiment was designed to elucidate the effects of encountering *N. pyrausta* spores (horizontal transmission), and Cry1Ab-incorporated diet in resistant and susceptible *O. nubilalis*. Late-instar (3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup>) *O. nubilalis*, resistant and susceptible to Cry1Ab, were exposed to Cry1Ab-incorporated or non-Bt diet treated or untreated with *N. pyrausta* spores, and assessed at 7 and 14 days. Survival and development were evaluated at 14 days, change in weight at 7 days; neither Cry1Ab-incorporated diet nor encountering *N. pyrausta* spores reduced survival of resistant larvae. However, development and weight gain of resistant larvae were slowed when feeding on Cry1Ab-incorporated diet, and Cry1Ab-incorporated diet treated with *N. pyrausta*, but not on non-Bt diet treated with *N. pyrausta*. Survival, development, and weight of susceptible larvae were negatively impacted when feeding on Cry1Ab, but not non-Bt diet with *N. pyrausta*. The combination of *N. pyrausta* spores and Cry1Ab-incorporated diet killed all susceptible larvae. This experiment demonstrates that susceptible late-instars encountering Cry1Ab-incorporated diet and spores of *N. pyrausta* will not survive. This microsporidium, indigenous in *O. nubilalis* populations, may play a role in resistance management by reducing survival of larvae on Bt corn.

---

<sup>1</sup> Graduate student, Adjunct Assistant Professor, Adjunct Professor, respectively, Department of Entomology, Iowa State University. USDA-ARS, Ames, Iowa.

<sup>2</sup> Primary researcher and author

## INTRODUCTION

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is a major pest of corn in the United States and other countries (Mason et al., 1996). *Ostrinia nubilalis* undergo complete metamorphosis with 5 instars. Damage to corn can be attributed to the larval stage; adult *O. nubilalis* lay their eggs on corn leaves, and the larvae eventually bore into the stalk. This tunneling decreases the number of vascular bundles in the stalk, causing damage due to disruption of water and nutrient movement throughout the plant (Mason et al., 1996). Larvae can also cause yield loss by feeding directly on ears. Costs associated with yield loss and control measures for *O. nubilalis* exceed \$1 billion annually. Many tactics have been used to manage *O. nubilalis* including cultural controls, e.g. burning residue, host plant resistance, chemical insecticides, biological control, and recently transgenically modified corn hybrids. One organism that helps to regulate *O. nubilalis* populations is *Nosema pyrausta* Paillot (Microsporidia: Nosematidae). *Nosema pyrausta* is an obligate intracellular parasite of *O. nubilalis*. This microsporidium infects *O. nubilalis* across its range, with varying infection intensities and percentages (Zimmack et al., 1954; Andreadis, 1982). Infection with *N. pyrausta* can cause decreased survival and fecundity, as well as delayed larval development and pupal deformities (Zimmack et al., 1954; Windels et al., 1976). Intensity of *N. pyrausta* infection directly impacts the severity of symptoms, low level infections may not have much influence on fecundity or survival, while high intensity infections have more dramatic impacts (Sajap and Lewis, 1988). *Nosema pyrausta* is transmitted from insect to insect both transovarially from female to offspring (vertical) (Zimmack and Brindley, 1957; Siegel et al., 1986; Sajap and Lewis, 1988), and through consumption of spores deposited in frass (horizontal) (Lewis, 1978; Andreadis, 1986; 1987). *Ostrinia nubilalis* larvae may become infected by consuming spores while feeding in close proximity to an infected larva (Andreadis, 1987), however they may also consume spores that have remained in the corn plant for a longer period of time, e.g. a second generation larva may become infected consuming frass left behind by a first generation larva (Lewis and Cossentine, 1986). *Nosema pyrausta* is generally chronic, but when combined with other stress factors such as weather (Lewis, 1975; Kramer,

1959), crowding (Siegel et al., 1986), or host-plant resistance (Lewis and Lynch, 1976; Lynch and Lewis, 1976), its impact is much stronger. Insecticides combined with *N. pyrausta* also work additively in suppressing *O. nubilalis* (Lublinkhof et al., 1979) as does Bt (Pierce et al., 2001; Reardon et al., 2004).

Transgenically modified Bt corn for control of *O. nubilalis* was first registered for use in 1996, and its adoption has increased since (USDA, 2007). In 2007, Bt corn made up 49% of the corn planted in the United States (USDA, 2007). *Ostrinia nubilalis* larvae that feed on Bt corn are sensitive to the Cry toxin produced within the plant, and are killed. One concern regarding the planting of Bt corn is that *O. nubilalis* will evolve resistance to the Cry toxin thus rendering the technology less effective. To date, there are no reports of insects evolving high levels of resistance to Bt crops in the field, and no reports of crop failure due to Bt resistance, however several insect species have laboratory-selected Bt resistance, including Cry1Ab-resistant *O. nubilalis* (Sumerford et al., 2008). The threat of natural populations of *O. nubilalis* developing resistance to Bt corn has led to adoption of a resistance management program, the high dose/refuge strategy. This tactic is two-fold; it requires that the level of toxin expressed within the plant be high enough to kill 99.9% of individuals heterozygous for a resistance allele, and that a specified portion of the crop (currently 20% in the corn belt) be planted to a non-Bt refuge. This resistance management strategy assumes that resistance alleles are rare within the population. The refuge theoretically produces a cohort of susceptible adults to mate with potentially resistant adults thereby preventing production of homozygous-resistant offspring.

A study conducted by Walker et al. (2000) indicated differential survival of Bt-susceptible *O. nubilalis* instars on Bt transgenic corn. In another study, late-instar Bt-susceptible *O. nubilalis* exposed to Dipel® (a commercial formulation of Bt) had greater survival at five days than earlier instars (Huang et al., 1999). Late-instar *O. nubilalis* larvae are known to move between plants, especially if the plants they are residing in are destroyed (Reardon et al., 2007). Cry1Ab-resistant, neonatal *O. nubilalis* that are vertically-infected with *N. pyrausta*, are known to have greater mortality and slower development on Cry1Ab-incorporated diet compared with non-infected, resistant larvae

(See Ch. 2, 4), but effects of Cry1Ab on horizontally-infected resistant *O. nubilalis* have not been studied. The objective of this experiment was to measure survival and development of late-instar (3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup>) *O. nubilalis* that began their development on non-Bt diet and were transferred to Cry1Ab-incorporated diet topically-treated or untreated with *N. pyrausta* spores.

## MATERIALS AND METHODS

**Insect Preparation.** Two populations of *O. nubilalis* were used, both from the USDA Corn Insects and Crop Genetics Research Unit in Ames, Iowa; a susceptible population that is continuously reared, and a laboratory-selected Cry1Ab-resistant population. The resistant population exhibits 2500x resistance compared to the susceptible population (Sumerford et al., 2008). Hereafter, they will be referred to as resistant and susceptible. Eggs from both populations were hatched into small containers (8 cm diameter) (Pioneer Plastics, Inc., Dixon, KY), of non-Bt meridic diet (Guthrie et al., 1985) for several successive days, and maintained at ideal larval conditions of 27°C, 70% relative humidity (Rh), and continuous light. At the onset of experimentation, resistant and susceptible 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars were selected from the containers and any clinging diet or frass was removed. Larvae were weighed and placed into empty 18.5 ml plastic cups (Anderson Tool and Die, Linden, NJ) to be randomly assigned to a diet/*N. pyrausta* treatment. Instar was determined using head capsule size (DeWitt and Stockdale, 1983). In addition to head capsule size a criterion for selection within 5<sup>th</sup> instars required the weight to be less than 50 g to prevent using more developed larvae that would pupate immediately rather than feed on the diet.

**Diet Preparation.** Two meridic diets were prepared, one incorporated with Cry1Ab toxin (trypsin-activated Cry1Ab; obtained from Dr. M. Carey, Dept. of Biochemistry, Case Western Reserve University, Cleveland, OH) at a rate of 10 µg/ml, and one without Cry1Ab. Neither diet contained Fumidil B which inhibits reproduction of *N. pyrausta* (Lewis and Lynch, 1970). While still liquid, the diets were metered into 18.5 ml plastic cups, 5 ml per cup so that surface area would be equivalent among cups. Great care was taken to prevent bubbles, divets, or other surface imperfections on the diet surfaces which would concentrate the *N. pyrausta* spores. The diet cups were further

separated into 2 groups, one half was surface-treated with *N. pyrausta* spores, the remainder receiving a control treatment. *Nosema pyrausta* spores were harvested from frozen larval *O. nubilalis* cadavers from a highly infected population. The cadavers were homogenized in a blender with water; the resulting solution was filtered and diluted to a final concentration to equal 1000 spores/mm<sup>2</sup> of diet. A small amount (.01 mg/ml water) of chlorotetracycline (64 g/lb) was added to the spore solution to prevent bacterial growth. On the day of experimentation, .2 ml of *N. pyrausta* spore solution was pipetted onto the surfaces of half of the Cry1Ab-incorporated diet cups and half of the non-Bt cups. The remaining cups were treated with .2 ml of a control solution containing an equivalent concentration of chlorotetracycline (64 g/lb). After each tray of 30 cups was inoculated with the appropriate solution, the trays were tilted in all directions to swirl the liquid ensuring even coverage across the diet surfaces. Diet cups were air-dried before larvae were introduced.

**Table 1: Experimental treatments; numbers refer to treatment, all combinations of population, *N. pyrausta*, diet, and instar total 24 treatments.**

<i>Population</i>	<i>N. pyrausta</i>	<i>Diet</i>	<i>3<sup>rd</sup> Instar</i>	<i>4<sup>th</sup> Instar</i>	<i>5<sup>th</sup> Instar</i>
Resistant	Untreated	Non-Bt	1	2	3
Resistant	Untreated	Bt	4	5	6
Resistant	<i>N. pyrausta</i>	Non-Bt	7	8	9
Resistant	<i>N. pyrausta</i>	Bt	10	11	12
Susceptible	Untreated	Non-Bt	13	14	15
Susceptible	Untreated	Bt	16	17	18
Susceptible	<i>N. pyrausta</i>	Non-Bt	19	20	21
Susceptible	<i>N. pyrausta</i>	Bt	22	23	24

**Experimental Design and Analysis.** A total of 24 treatments included all combinations of population (resistant or susceptible), instar (3<sup>rd</sup>, 4<sup>th</sup>, or 5<sup>th</sup>), diet (Cry1Ab-incorporated or control), and *N. pyrausta* (diet treated or untreated with *N. pyrausta* spores). The treatments were randomly assigned in six blocks, five larvae per treatment (See Table 1). Data were recorded at 7 (survival and change in weight) and 14 (survival and development) days. To enhance normality and homogenize variances, data on weight differences were Log<sub>10</sub>-transformed. Proc Mixed of SAS (v. 9.1.3) was used to perform the analysis. Separate analyses were conducted for each instar, including

Analysis of Variance tests of fixed effects, and means separation, method=Tukey-Kramer.

## RESULTS

Survival data were recorded 14 days after placement into arenas. Treatments were different with regards to survival in all 3 instars (Table 2). Encountering Bt diet and spores of *N. pyrausta* affected survival of the susceptible, but not the resistant populations. Survival of 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> resistant instars that were transferred to Cry1Ab-incorporated diet did not differ significantly from those transferred to non-Bt diet (Fig. 1). When resistant instars encountered *N. pyrausta* spores on control diet, survival was not significantly different from those on control diet without *N. pyrausta* spores. The combination of Cry1Ab-incorporated diet and spores of *N. pyrausta* had no effect on 14 day-survival of resistant larvae. In contrast, susceptible instars transferred to Cry1Ab-incorporated diet experienced significantly reduced survival compared with larvae transferred to control diet. Encountering *N. pyrausta* spores alone did not decrease survival, but the combination of *N. pyrausta* spores and Cry1Ab-incorporated diet caused 100% mortality of susceptible instars.

**Table 2: Analysis of Variance for three parameters: 14 day survival, 7 day change in weight, and proportion of survivors pupated at 14 days.**

	<i>NDF, DDF</i>	<i>F</i>	<i>P</i>
<b>Survival</b>			
3 <sup>rd</sup> Instar	7, 35	148.86	<.0001
4 <sup>th</sup> Instar	7, 35	62.89	<.0001
5 <sup>th</sup> Instar	7, 35	16.40	<.0001
<b>7 day weight change</b>			
3 <sup>rd</sup> Instar	7, 33	167.69	<.0001
4 <sup>th</sup> Instar	7, 35	389.49	<.0001
5 <sup>th</sup> Instar	7, 35	217.47	<.0001
<b>Proportion pupae of survivors</b>			
3 <sup>rd</sup> Instar	6, 29	45.55	<.0001
4 <sup>th</sup> Instar	6, 30	39.39	<.0001
5 <sup>th</sup> Instar	6, 29	90.85	<.0001

Larvae were weighed prior to placement in arenas and after 7 days; the difference in Log<sub>10</sub>-transformed weight was used to measure the effects of treatment on larval development. ANOVA showed that for change in weight, treatments were different in all

3 instars (Table 2). Cry1Ab-incorporated diet significantly slowed weight gain in resistant instars, but encountering *N. pyrausta* spores alone did not affect weight gain at 7 days (Fig. 2). Resistant 3<sup>rd</sup> and 4<sup>th</sup> instars transferred to Cry1Ab-incorporated diet without *N. pyrausta* spores gained significantly less weight compared to those on non-Bt diet. However, there was no significant difference in weight gain in resistant 5<sup>th</sup> instars transferred to Cry1Ab-incorporated diet compared with those transferred to non-Bt diet. Encountering *N. pyrausta* spores alone did not affect weight of 3<sup>rd</sup>, 4<sup>th</sup>, or 5<sup>th</sup> resistant instars. The combined effect of encountering Cry1Ab-incorporated diet and *N. pyrausta* spores did not significantly differ from Cry1Ab-incorporated diet alone in 3<sup>rd</sup> and 5<sup>th</sup> resistant instars, however 4<sup>th</sup> instars gained less weight on Cry1Ab-incorporated diet treated with *N. pyrausta* spores compared with untreated Cry1Ab-incorporated diet. Resistant 5<sup>th</sup> instars that encountered Cry1Ab-incorporated diet in combination with *N. pyrausta* spores gained significantly less weight than those on non-Bt diet with or without *N. pyrausta* spores, but this value did not differ significantly from 5<sup>th</sup> instars on Cry1Ab-incorporated diet alone. Susceptible 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars had lost weight 7 days after being transferred to Cry1Ab-incorporated diet (Fig. 2). Encountering spores of *N. pyrausta* on regular diet did not affect weight of any susceptible instar. The combination of transfer to Cry1Ab-incorporated diet and encountering *N. pyrausta* spores did not decrease weight further than transfer to Cry1Ab-incorporated diet alone.

Development delays were assessed by calculating the proportion of survivors that had pupated at 14 days. The percentage of pupae differed significantly among treatments in all instars (Table 2). Transfer to Cry1Ab-incorporated diet slowed development of resistant 3<sup>rd</sup> and 4<sup>th</sup> instars (Fig. 3). Encountering *N. pyrausta* spores on regular diet did not significantly affect development of any resistant instars. *Nosema pyrausta* spores in combination with Cry1Ab-incorporated diet slowed development of resistant 4<sup>th</sup> and 5<sup>th</sup> instars compared with Cry1Ab-incorporated diet alone. Even though resistant 5<sup>th</sup> instars were not significantly delayed when transferred to untreated Cry1Ab-incorporated diet, encountering Cry1Ab-incorporated diet treated with *N. pyrausta* spores did cause a significant developmental delay. Development of susceptible 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars was strongly delayed on Cry1Ab-incorporated diet compared with those on non-Bt diet.

Encountering *N. pyrausta* spores on non-Bt diet had no effect on proportion of larvae that had pupated at 14 days. Susceptible larvae that were transferred to Cry1Ab-incorporated diet treated with *N. pyrausta* spores did not survive.

## DISCUSSION

This experiment was undertaken to investigate two issues; 1) how feeding on Cry1Ab-incorporated diet affects survival and growth of late-instar resistant and susceptible *O. nubilalis*, and 2) whether encountering *N. pyrausta* affects survival and growth of the same on Cry1Ab-incorporated and non-Bt diets. Survival of resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars was not significantly reduced at 14 days when feeding on Cry1Ab-incorporated diet, encountering *N. pyrausta* spores, or a combination of the two (Fig. 1). However, resistant *O. nubilalis* feeding on Cry1Ab-incorporated diet were developmentally delayed and gained less weight than those on non-Bt diet. From previous experimentation it is known that resistant larvae that begin feeding on Cry1Ab-incorporated diet as neonates are developmentally-delayed (See Ch. 2). The same trend was apparent when late instars were exposed to Cry1Ab-incorporated diet. The larvae were developmentally delayed, but their survival at 14 days was not affected, and they still gained weight, although at a slower rate.

Late instar susceptible larvae experienced reduced survival when feeding on Cry1Ab-incorporated diet (Fig. 1). Huang et al. (1999) performed a study exposing *O. nubilalis* to Dipel® (a commercial Bt formulation), and noted higher survival of late instar *O. nubilalis* compared with early instars after 5 days. While there were some susceptible larvae still living at 14 days, they were developmentally-delayed (Fig. 2) and had lost weight after 7 days on Cry1Ab-incorporated diet (Fig. 3). These results suggest that although they survived 14 days on Cry1Ab-incorporated diet, they were still strongly affected. Because susceptible larvae lost weight and were developmentally delayed, it is unlikely they would survive to adulthood on Cry1Ab-incorporated diet. Encountering *N. pyrausta* spores in combination with Cry1Ab-incorporated diet killed all susceptible 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars. Because *N. pyrausta* is not a highly virulent microsporidium, its effects are generally chronic, suppressing populations but not decimating them. However, when *O. nubilalis* larvae are subjected to additional stresses, the impact of *N.*

*pyrausta* is magnified. When resistant and susceptible larvae encountered *N. pyrausta* in the absence of Cry1Ab, there was no measurable disadvantage at 7 or 14 days. The response to *N. pyrausta* in combination with Cry1Ab-incorporated diet was more severe in the susceptible population (100% mortality) than the resistant population. Other studies have shown increased susceptibility to Bt in *N. pyrausta*-infected *O. nubilalis* (Ch. 2, 4; Pierce et al., 2001; Reardon et al., 2004).

Most *O. nubilalis* will be exposed to Bt corn as neonates upon hatching, and will succumb soon after initiating feeding. Late-instar larvae however are known to migrate between plants, but generally do not move farther than 1 row (.76 m) (Andreadis, 1986; Ross and Ostlie, 1990; Reardon et al., 2007). If refuge corn was planted in close proximity to transgenic, as would be the case with mixed refuge, the likelihood of migration from non-Bt to Bt plants would increase, and with it the probability of late-instars encountering Bt. *Nosema pyrausta*-infected larvae are just as likely to migrate to adjacent plants as uninfected larvae (Lewis, 1978). Horizontal transmission of *N. pyrausta* can occur when a larva migrates to and feeds on a spore-contaminated plant where an infected larva is feeding or has fed. *Nosema pyrausta* can also be transmitted per os between generations, as was shown by Lewis and Cossentine (1986), through consumption of frass remaining on the plant. If the results of this study represent the effects on a natural population in transgenic corn, susceptible larvae that become infected with *N. pyrausta* on non-Bt corn would have a high probability of survival, but once they migrated to a Bt plant that probability would be reduced. Mortality of susceptible larvae in response to encountering Cry1Ab-incorporated diet and *N. pyrausta* spores could increase the likelihood of resistant-resistant mating in *O. nubilalis* by lowering the number of available susceptible mates.

While survival of resistant late-instars feeding on Cry1Ab-incorporated diet was not reduced at 14 days, development of 3<sup>rd</sup> and 4<sup>th</sup> instars was slowed, and weight gain of 3<sup>rd</sup> and 4<sup>th</sup> instars at 7 days was less than those on non-Bt diet. Resistant 4<sup>th</sup> and 5<sup>th</sup> instars that were transferred to Bt diet treated with *N. pyrausta* spores were developmentally delayed to a greater extent than those encountering Bt diet alone. In resistant larvae, increased developmental periods would allow more time for *N. pyrausta*

to reproduce, leading to a more intense infection. The impact of infection with *N. pyrausta* is time-dependant, and when the larva became infected and the number of spores consumed impacts severity, with earlier exposure and higher spore consumption negatively influencing survival and fecundity (Sajap and Lewis, 1992). Vertically infected *O. nubilalis* experience greater mortality due to *N. pyrausta* infection than horizontally infected *O. nubilalis* (Solter et al., 1990). Cry1Ab-resistant neonatal *O. nubilalis* larvae, vertically-infected with *N. pyrausta*, experienced greater mortality and slower growth on Cry1Ab-incorporated diet and Bt tissue-incorporated diet than uninfected larvae (See Ch. 2, 4). However, developmental delays caused by feeding on Cry1Ab-incorporated diet create a situation in which *N. pyrausta* infection can intensify to lethal levels. Given this, it is unlikely that even resistant larvae would be able to complete their development on Bt plants if also infected with *N. pyrausta*. Because even 5<sup>th</sup> instar resistant and susceptible *O. nubilalis* were significantly impacted by *N. pyrausta* on Bt, it appears there is a large window of opportunity to infect *O. nubilalis*. Migrating larvae can disseminate *N. pyrausta* spores leading to infection in larvae in adjacent plants, and this phenomenon has been shown to reduce larval densities on non-Bt plants (Lewis, 1978). Because susceptible larvae encountering *N. pyrausta* and Cry1Ab-incorporated diet were killed, horizontal transmission of *N. pyrausta* could potentially eliminate susceptible *O. nubilalis* on Bt plants. Planting of mixed refuge increases the likelihood of susceptible larvae migrating from non-Bt to Bt plants, and infected susceptible late-instars moving to Bt plants would most likely be killed. From a damage control standpoint this could be considered a benefit, however the purpose of the refuge is production of susceptible mates, and horizontal transmission and migration between plants may reduce survival of susceptible *O. nubilalis*. *Nosema pyrausta* is indigenous in *O. nubilalis* populations (Hill and Gary, 1979) and has been shown to have increased detrimental impacts to susceptible and resistant *O. nubilalis* in combination with Cry1Ab-incorporated diet. Because *N. pyrausta* has the potential to influence evolution of resistance to Bt in *O. nubilalis*, it should be factored into decision making with regards to resistance management.

**FIGURE CAPTIONS**

- Figure 1. Percentage survival at 14 days of susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars transferred to Bt or Regular (non-Bt) diet treated or untreated with *N. pyrausta* spores (Np +, Np -). Least-squares means comparison: Method=Tukey-Kramer (P<0.05) used to generate estimates. Within an instar, estimates labeled with the same letter are not significantly different at the P .05 level.
- Figure 2. Log<sub>10</sub>-transformed change in weight at 7 days of susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars transferred to Bt or Regular (non-Bt) diet treated or untreated with *N. pyrausta* spores (Np +, Np -). Least-squares means comparison: Method=Tukey-Kramer (P<0.05) used to generate estimates. Within an instar, estimates labeled with the same letter are not significantly different at the P .05 level.
- Figure 3. Development as measured by the proportion of survivors that had pupated at 14 days of susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars transferred to Bt or Regular (non-Bt) diet treated or untreated with *N. pyrausta* spores (Np +, Np -). Least-squares means comparison: Method=Tukey-Kramer (P<0.05) used to generate estimates. Within an instar, estimates labeled with the same letter are not significantly different at the P .05 level. Susceptible 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars transferred to Cry1Ab-incorporated diet treated with *N. pyrausta* spores were not included in analysis because there were no survivors at 14 days.

Fig. 1

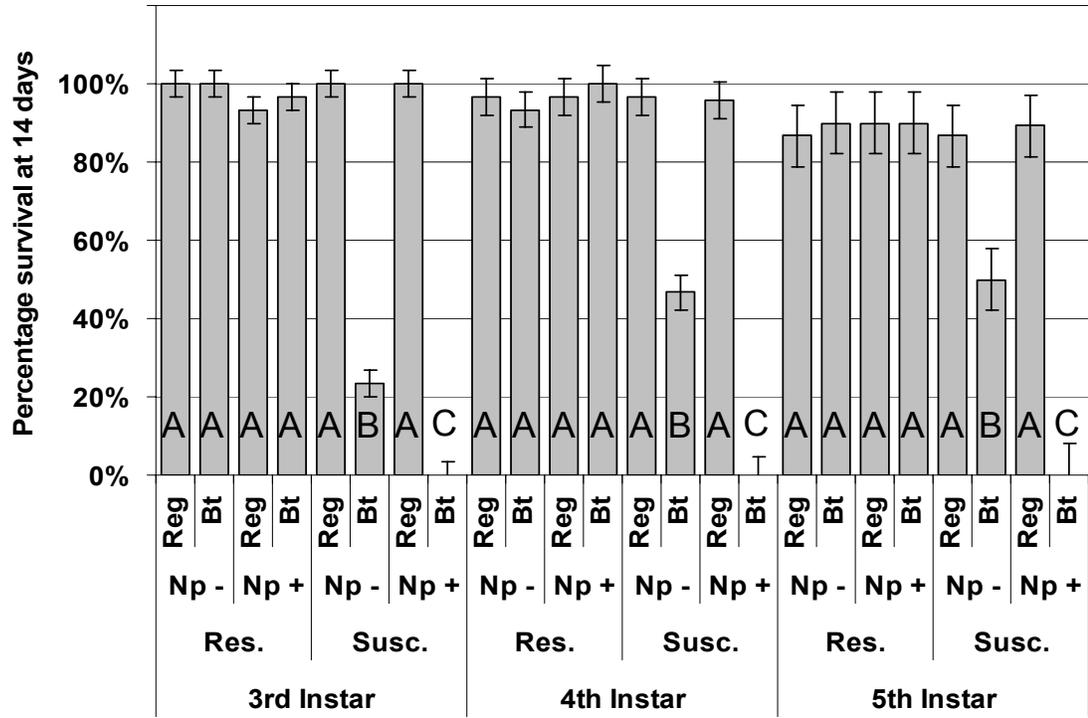


Fig. 2

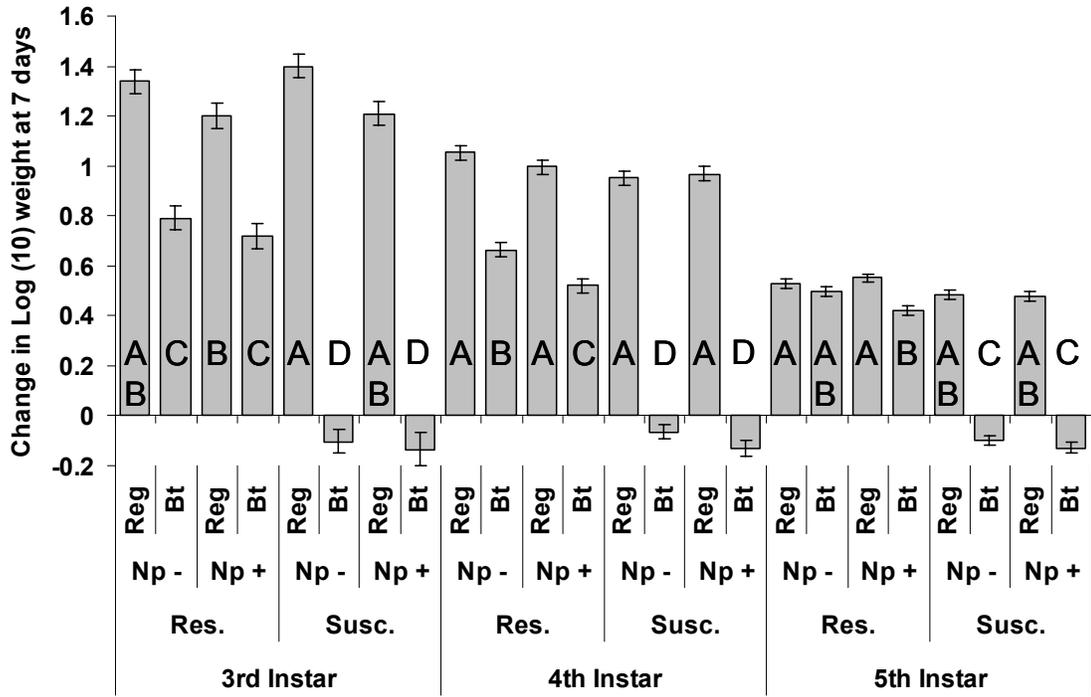
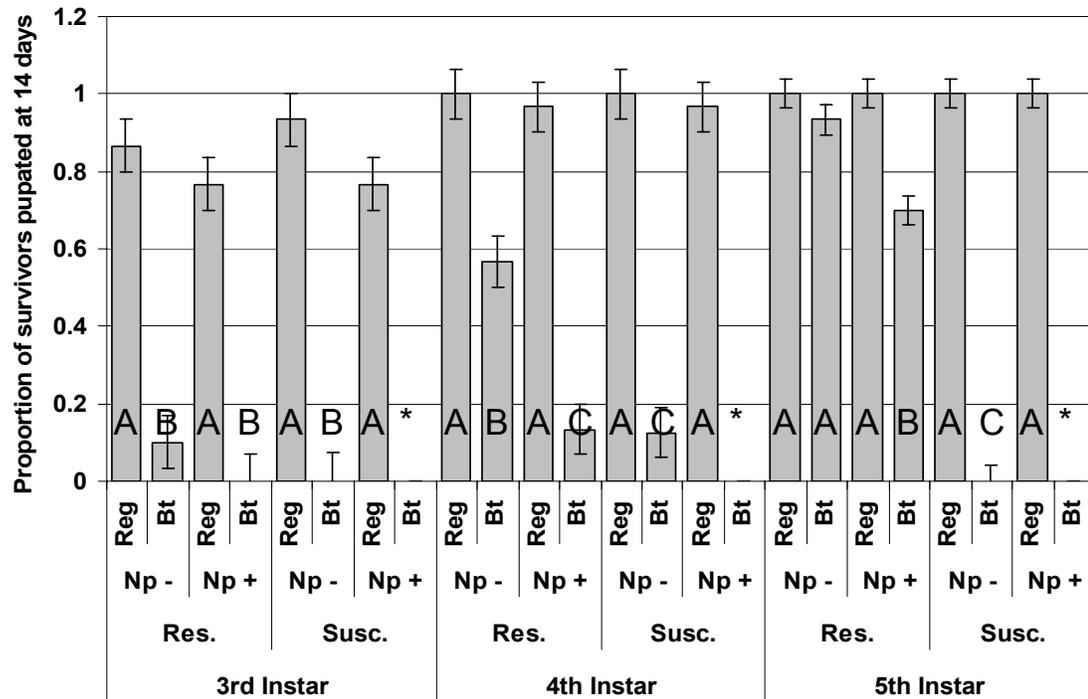


Fig. 3



### ACKNOWLEDGEMENTS

We thank J Robbins, R Gunnarson, D Steines, and J Dyer.

### REFERENCES

- Andreadis, T. G. 1982.** Impact of *Nosema pyrausta* on field populations of *Macrocentrus grandii*, an introduced parasite of the European corn borer, *Ostrinia nubilalis*. *J. Invert. Pathol.* 39: 298-302.
- Andreadis, T. G. 1986.** Dissemination of *Nosema pyrausta* in feral populations of the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 48: 335-343.
- Andreadis, T. G. 1987.** Horizontal transmission of *Nosema pyrausta* (Microsporida: Nosematidae) in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Environ. Entomol.* 16: 1124-1129.
- DeWitt, J., and H. Stockdale. 1983.** Field crop insect stages. Pm-953. Cooperative Extension Service. Iowa State University.

- Guthrie, W. D., J. C. Robbins, and J. L. Jarvis. 1985.** *Ostrinia nubilalis*, pp. 407-413. In P. Singh & R.F. Moore [eds.], Handbook of insect rearing, vol. 2. Elsevier, New York.
- Hill, R. E., and W. J. Gary. 1979.** Effects of the microsporidium, *Nosema pyrausta*, on field populations of European corn borers in Nebraska. Environ. Entomol. 8: 91-95.
- Huang, F., L. L. Buschman, and R. A. Higgins. 1999.** Susceptibility of different instars of European corn borer (Lepidoptera: Crambidae) to diet containing *Bacillus thuringiensis*. J. Econ. Entomol. 92: 547-550.
- Kramer, J. P. 1959.** Observations on the seasonal incidence of microsporidiosis in European corn borer populations in Illinois. Entomophaga. 4: 37-42.
- Lewis, L. C. 1975.** Natural regulation of crop pests in their indigenous ecosystems and in Iowa agroecosystems: bioregulation of economic insect pests. Iowa State J. Res. 49: 435-445.
- Lewis, L. C. 1978.** Migration of larvae of *Ostrinia nubilalis* (Lepidoptera: Pyralidae) infected with *Nosema pyrausta* (Microsporida: Nosematidae) and subsequent dissemination of this microsporidium. Can. Ent. 110: 897-900.
- Lewis, L. C., and R. E. Lynch. 1970.** Treatment of *Ostrinia nubilalis* larvae with Fumidil B to control infections caused by *Perezia pyraustae*. J. Invertebr. Pathol. 15: 43-48.
- Lewis, L. C., and R. E. Lynch. 1976.** Influence on the European corn borer of *Nosema pyrausta* and resistance in maize to leaf feeding. Environ. Entomol. 5: 139-142.
- Lewis, L. C., and J. E. Cossentine. 1986.** Season long intraplant epizootics of entomopathogens, *Beauveria bassiana* and *Nosema pyrausta*, in a corn agroecosystem. Entomophaga. 31: 363-369.
- Lublinkhof, J., L. C. Lewis, and E. C. Berry. 1979.** Effectiveness of integrating insecticides with *Nosema pyrausta* for suppressing populations of the European corn borer. J. Econ. Entomol. 72: 880-883.
- Lynch, R.E., and L. C. Lewis. 1976.** Influence on the European corn borer of *Nosema pyrausta* and resistance in maize to sheath-collar feeding. Environ. Entomol. 5: 143-146.
- Mason, C. E., M. E. Rice, D. D. Calvin, J. W. Van Duyn, W. B. Showers, W. D. Hutchison, J. F. Witkowski, R. A. Higgins, D. W. Onstad, and G. P. Dively.**

- 1996.** European corn borer ecology and management. North Central Regional Extension Pub. 327.
- Pierce, C. M. F., L. F. Solter, and R. A. Weinzierl. 2001.** Interactions between *Nosema pyrausta* (Microsporidia: Nosematidae) and *Bacillus thuringiensis* subsp. *kurstaki* in the European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 94: 1361-1368.
- Reardon, B. J., R. L. Hellmich, D. V. Sumerford, and L. C. Lewis. 2004.** Growth, development, and survival of *Nosema pyrausta*-infected European corn borers (Lepidoptera: Crambidae) reared on meridic diet and Cry1Ab. *J. Econ. Entomol.* 97: 1198-1201.
- Reardon, K. T., R. L. Hellmich, D. V. Sumerford, L. C. Lewis, B. J. Reardon, and D. D. Calvin. 2007.** Influence of plant severing on movement of *Ostrinia nubilalis* larvae in *Zea mays* hybrid seed production fields. *J. Econ. Entomol.* 100: 1129-1135
- Ross, S. E., and K. R. Ostlie. 1990.** Dispersal and survival of early instars of European corn borer (Lepidoptera: Pyralidae) in field corn. *J. Econ. Entomol.* 83: 831-836.
- Sajap, A. S., and L. C. Lewis. 1988.** Histopathology of transovarial transmission of *Nosema pyrausta* in the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 52: 147-153.
- Sajap, A. S., and L. C. Lewis. 1992.** Chronology of infection of European corn borer (Lepidoptera: Pyralidae) with the microsporidium *Nosema pyrausta*: effect on development and vertical transmission. *Environ. Entomol.* 21: 178-182.
- SAS Institute. 2004.** (Version 9.1.3), SAS Institute, Cary, NC.
- Siegel, J. P., J. V. Maddox, and W. G. Ruesink. 1986.** Lethal and sublethal effect of *Nosema pyrausta* on the European corn borer (*Ostrinia nubilalis*) in Central Illinois. *J. Invertebr. Pathol.* 48: 167-173.
- Solter, L. F., J. V. Maddox, and D. W. Onstad. 1990.** Transmission of *Nosema pyrausta* in adult European corn borers. *J. Invertebr. Pathol.* 57: 220-226.
- Sumerford, D. V., L. C. Lewis, and J. C. Robbins. 2008.** Inheritance of resistance to Cry1Ab in a laboratory-selected colony of *Ostrinia nubilalis*: patterns of larval survival and larval development. Accepted to *Heredity*.
- USDA, Economic Research Service. 2007.** Adoption of Genetically Engineered Crops in the U.S.: Extent of Adoption. 5 July 2007. Content accessed 3/13/2008. <http://www.ers.usda.gov/Data/BiotechCrops/adoption.htm>.

- Walker, K. A., R. L. Hellmich, and L. C. Lewis. 2000.** Late-instar European corn borer (Lepidoptera: Crambidae) tunneling and survival in transgenic corn hybrids. *J. Econ. Entomol.* 93: 1276-1285.
- Windels M. B., H. C. Chiang, and B. Furgala. 1976.** Effects of *Nosema pyrausta* on pupa and adult stages of the European corn borer *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 27: 239-242.
- Zimmack, H. L., K. D. Arbuthnot, and T. A. Brindley. 1954.** Distribution of the European corn borer parasite *Perezia pyraustae* and its effect on the host. *J. Econ. Entomol.* 47: 641-645.
- Zimmack, H. L., and T. A. Brindley. 1957.** The effect of the protozoan parasite *Perezia pyraustae* Paillot on the European corn borer. *J. Econ. Entomol.* 50: 637-640.

## CHAPTER 6. GENERAL CONCLUSIONS

### GENERAL CONCLUSIONS

Transgenically-modified Bt corn is a very effective pest management tool, and is being planted in greater amounts each year. However, there is concern that *Ostrinia nubilalis* may evolve resistance to Bt corn, rendering it ineffective. The microsporidium *Nosema pyrausta* is indigenous in *O. nubilalis* populations, and this thesis reports investigative research on effect of *N. pyrausta* in combination with Bt on susceptible and Cry1Ab-resistant *O. nubilalis*. In general, *N. pyrausta* increased mortality of *O. nubilalis* on Bt compared to non-infected *O. nubilalis*.

In the experiment designed to measure development, it was found that Cry1Ab-resistant *O. nubilalis* developed more slowly on Cry1Ab-incorporated diet than susceptible *O. nubilalis* on non-Bt diet. If this same phenomenon occurs in fields of transgenic Bt corn, late emergence of resistant *O. nubilalis* may mean that the high dose/refuge strategy assumption of random mating will not be met. Assortative mating of resistant *O. nubilalis* would hasten the evolution of resistance by increasing the frequency of resistance alleles in the population. However, susceptible *O. nubilalis* feeding on regular diet that were infected with *N. pyrausta* were delayed to a similar extent as resistant *O. nubilalis* on Cry1Ab-incorporated diet. Rather than fully-assortative mating among resistant adults, resistant adults may instead mate with *N. pyrausta*-infected susceptible adults because of their respective developmental delays.

Further experiments were conducted to examine the effect of *N. pyrausta* and Bt on offspring resulting from susceptible-resistant mating, and resistant-resistant mating. If a resistant male mates with an infected susceptible female, their offspring will be partially-resistant and may be infected with *N. pyrausta*, because infected females vertically transmit *N. pyrausta*. These larvae will be unlikely to survive on Bt, and their resistance allele(s) will not continue to proliferate. Because *N. pyrausta* and Bt delay larval development, resistant *O. nubilalis* that were both infected with *N. pyrausta* and feeding on Bt were delayed to a greater extent than is caused by either factor alone. In a transgenic Bt field, if an infected resistant *O. nubilalis* did manage to complete its

development on Bt, it would most likely emerge after the refuge population had completed mating. Because survival to adulthood was very low for resistant infected *O. nubilalis* on Bt, it is unlikely that sufficient numbers of adults would be present, reducing their ability to locate one another and mate successfully. However, because a resistant infected population emerging from Bt would likely be completely temporally isolated from the refuge population, assortative mating could occur. The infected offspring that would result would also be unlikely to survive on Bt. These experiments illustrate the potential of *N. pyrausta* to delay evolution of resistance to Bt by killing resistant *O. nubilalis* on Bt, thus removing them as contributors of resistance alleles to subsequent generations.

*Nosema pyrausta* in combination with Bt reduced survival of susceptible, partially-resistant, and fully-resistant *O. nubilalis* that were vertically-infected and exposed to Bt as neonates. It has been hypothesized that if *O. nubilalis* are not exposed to Bt until they are late-instars that they will be able to survive to adulthood, selecting for low-level resistance. The experiment designed to observe the impacts of Bt and *N. pyrausta* on late-instars exposed uninfected susceptible and resistant 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars to Bt or non-Bt diet that had been treated or untreated with *N. pyrausta* spores. Encountering *N. pyrausta* spores on non-Bt diet did not affect survival or development of susceptible and resistant *O. nubilalis*. Resistant *O. nubilalis* that encountered Bt diet did not have reduced survival, but their weight and development were impacted compared to those on non-Bt diet. Encountering a combination of Bt diet and *N. pyrausta* spores did not reduce survival to a greater extent than Bt diet alone, but development was slowed. Susceptible *O. nubilalis* that were exposed to Bt diet lost weight and had reduced survival, and susceptible *O. nubilalis* that encountered both *N. pyrausta* spores and Bt diet were all killed. This experiment provides two useful pieces of information regarding late-instar movement in transgenic corn; 1) late-instar susceptible and resistant larvae were strongly impacted on Bt diet, and 2) encountering *N. pyrausta* in combination with Bt killed susceptible larvae. Horizontal transmission of *N. pyrausta* occurs in fields of corn, and can proliferate infection on a small scale.

*Nosema pyrausta* may be present in all life stages of *O. nubilalis*. This thesis examined the impact of *N. pyrausta* and Bt in vertically-infected *O. nubilalis* as well as those that obtained infection as 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars. The impact of adult infection with *N. pyrausta* on offspring was also examined. Because *N. pyrausta* impacts survival on Bt at various stages of *O. nubilalis* development, any chance to encounter *N. pyrausta* may have some benefit in delaying resistance evolution. *Ostrinia nubilalis* living in refuge corn provide *N. pyrausta* inoculum in three ways: 1) larvae deposit frass containing viable spores that can infect additional larvae feeding on the same plant, 2) infected larvae move between plants, spreading *N. pyrausta* to larvae on additional plants, and 3) infected females oviposit vertically-infected eggs, establishing new foci of infections. This thesis has demonstrated a benefit of infection with *N. pyrausta* for resistance management. Corn that is planted as refuge to produce susceptible mates may also be providing a source of *N. pyrausta* inoculum, as *O. nubilalis* living in the refuge constitute a reservoir for *N. pyrausta*.

#### **FUTURE RESEARCH DIRECTIONS**

In general, *O. nubilalis* larvae that are infected with *N. pyrausta* are more susceptible to feeding on Bt, whether they are Bt-susceptible or Bt-resistant. This microsporidium is indigenous in *O. nubilalis* populations, but at varying levels. Enhancement or augmentation of *N. pyrausta* within *O. nubilalis* populations may increase susceptibility to Bt, and therefore decrease the likelihood of resistant or susceptible *O. nubilalis* surviving on Bt corn. Because it is a living organism, traditional insecticide application methods are not applicable to *N. pyrausta*. *N. pyrausta* only reproduces within the host organism, so augmentation could be accomplished by release or placement of infected *O. nubilalis*. While new foci of *N. pyrausta* infections are the result of oviposition by infected females (Andreadis, 1986), horizontal transmission results in build-up and dissemination of infection within fields (Lewis et al., 2006). In an area at risk for resistance development, augmentation may be accomplished with releases of infected adults, or possibly placement of infected larvae into the whorls of plants.

In conclusion, the significance of this work can best be realized in the area of predictive modeling. Models utilize factors such as frequency of resistance alleles and

rate of gene flow to predict when insects will become resistant. Models predicting *O. nubilalis* resistance to Bt corn have not accounted for the impact of developmental delays in resistant *O. nubilalis* feeding on Bt. Delayed adult emergence of resistant individuals may lead to assortative, rather than random mating. Assortative mating within a resistant population would hasten resistance evolution by increasing the frequency of resistance alleles in the population. Another aspect of *O. nubilalis* ecology that has not been included in resistance evolution models is the impact of infection with *N. pyrausta*. This indigenous microorganism delays larval development and increases mortality of infected *O. nubilalis*. When infected *O. nubilalis* are exposed to Bt, they have higher mortality, and slower development than non-infected *O. nubilalis*. Because *N. pyrausta* increases mortality in combination with Bt in both susceptible and resistant *O. nubilalis*, its presence may slow resistance development.

#### REFERENCES

- Andreadis, T. G. 1986.** Dissemination of *Nosema pyrausta* in feral populations of the European corn borer, *Ostrinia nubilalis*. *J. Invertebr. Pathol.* 48: 335-343.
- Lewis, L. C., D. V. Sumerford, L. A. Bing, and R. D. Gunnarson. 2006.** Dynamics of *Nosema pyrausta* in natural populations of the European corn borer, *Ostrinia nubilalis*: a six-year study. *BioControl* 51: 627-642.