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A COMPARISON OF UNIVOLTINE AND MULTIVOLTINE EUROPEAN CORN BORER (*OSTRINIA NUBILALIS* HÜBNER): LIFE HISTORY CHARACTERS, BT TOXIN SUSCEPTIBILITY, PARASITOID IMPACT, AND POPULATION PATTERN

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by

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Abstract:

Among the various stalk boring insect attacking corn in the U.S., European corn borer (ECB), *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), alone is responsible for a $1.85 billion annual loss. Soon after its discovery (1914) the insects displayed ecotype or voltine diversity. ECB management and its cost may be influenced by the ecotype pattern within a region. Since 1996, the commercial use of transgenic corn hybrids reduced the uses of synthetic insecticides in corn fields dramatically. However, wider uses of *Bt* corn poses the risk of resistance development and threatens parasitoids of ECB. Several Integrated Resistance Management (IRM) practices were introduced to minimize this threat, but the lack of understanding of the biology and population dynamics of geographic ecotypes of ECB may hinder the success of the IRM program. This dissertation studies were design to compare life history parameters, *Bt* toxin susceptibilities, parasitoid impacts, and population patterns between univoltine and multivoltine ecotypes collected from Pennsylvania, USA. From 2002-2005 post-diapause ECB were field collected in central, north, and south Pennsylvania. Various populations were reared through several generations in the laboratory to determine differences between univoltine and multivoltine ecotypes. These research included life history parameters, *Bt* susceptibility and parasitoid impacts.

Co-occurring univoltine ECB required higher degree-days to complete its life cycle than bivoltine ECB. Post-diapause univoltine pupal weights were significantly higher than multivoltine pupal weights, whereas the pupal weights of non-diapause ECB (F_1) reared in controlled environmental conditions were not significantly different. This suggests a non-genetic basis of weight gain. No variation was observed in reproductive parameters.

No significant mortality differences were found between univoltine and multivoltine ECB when subjected to Cry1Ab and Cry1F *Bt* toxins. A 1.5- to 2- fold increase in susceptibility for the LC_{50}, LC_{95}, and LC_{99} was observed in univoltine exposed to Cry1Ab. Sub-lethal effects were not significant for Cry1Ab, however, severe growth inhibition was observed in ecotypes when exposed to Cry1F toxin. These minor variations in susceptibility are unlikely to affect resistance.

Four years of field and laboratory study strongly suggested that *M. cingulum* emergence was completely synchronized with the spring emergence of the multivoltine ecotype. Post-diapause univoltine populations face almost no impact from *M. cingulum* parasitism. Sex ratio differences observed in over-wintered ECB populations in the presence or absence of *M. cingulum* parasitism suggested differential parasitism between male and female larvae.

Overall, experimental outcomes from this research suggested that in areas of co-occurrence, variation in ECB ecotypes is due to the synergistic effects of seasonal degree days, host plant stage, and perhaps parasitoid pressure. Further genetic structure studies of multi ecotype populations across several geographic areas may elucidate the general applicability of these findings.

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$\ln(1/\text{observed value} - 1) = \alpha + \beta * \text{accumulated degree days}$

$\ln(1/\text{observed value} - 1) = \alpha + \beta\ln * \text{accumulated degree days}$

$\text{Predicted \% completion of pupation} = 1/[1 + \exp^{(\alpha + \beta * \text{accumulated degree days})}]$

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Dedication

I dedicate this dissertation to my parents Shams uz Zaman and Fatema Zaman for their ever ending encouragement for all the good achievements in my life.

Chapter 1.

Introduction/ Literature Review.

1.1 Introduction into North America and range expansion:

The European corn borer (ECB), *Ostrinia nubilalis* (Hubner), (Lepidoptera: Crambidae) was first noticed near Boston, Massachusetts in 1914 (Vinal 1917). In 1919, the species was discovered in areas near Lake Erie in New York (Felt 1919). It is not clear how European corn borer first entered North America, but earlier researcher believed that *O. nubilalis* populations in North America probably originated through multiple introductions as a result of broomcorn importation from Hungary and Italy between the year 1909 and 1914 (Smith 1920, McLaine, 1922). Immediately after its introduction, ECB in North America were demonstrated voltine diversity, and it was speculated that at least two voltine types were introduced into North America. Vinal (1917) determined the existence of a bivoltine form where as Felt (1919) reported the presence of univoltine form of species near Lake Erie and in western New York State (Felt 1919).

Shortly after its introduction *O. nubilalis* population spread gradually from southern Michigan to northern Ohio. By the end of 1938, *O. nubilalis* expanded its range as far west as the shoreline of Lake Michigan in Wisconsin. Today, *O. nubilalis* is still spreading westward and south in the United States. Presently its range extends into Canada, westward to the Rocky Mountains, and southward into Florida and New Mexico. *Ostrinia nubilalis* now exists in all but the seven most western states of the United States. *O. nubilalis* currently shows voltine diversity across its geographic range in the North America.

Across its geographic range *O. nubilalis* is exposed to many ecological conditions such as variable temperature, photoperiod, latitude, length of growing season, and host plant availability. Diapause induction, length of diapause, diapause termination, interaction between day length and temperature are quite variable among individuals in the species.

Differences in these life history traits are more apparent in species that migrated from north to south because of the critical photoperiod and the decreased latitude (Showers et al. 1975, Lees 1980). Usually geographic populations are often closely adapted to their own part of their geographic range. When a species spreads into a new range, it is mismatched with the new region's season as a result of its close phenological adaptation in their own geographic range (Lees 1980). To acclimatize to the new environment the species faces environmental resistance that influences the rate of dispersal and adaptation to the new range. In their earlier expansion in 1940s European corn borer faced the least resistance for invading toward the west in the vast U.S. corn-belt than north to south (Bergman et al.1975). Also the westward expansion of ECB is not affected by photoperiod because of the similar latitude. Summer degree-day accumulations would increase as the species moved towards west that may have aided the horizontal expansion. Palmer et al. (1985) found that the univoltine population of Ohio migrated at a rate of 19 km per year whereas after the appearance of a bivoltine population, expansion westward averaged 114 km per year. Conversely, dispersal from north to south was much slower because of the variable photoperiod and thermal accumulation (degree-day) (Arbuthnot 1944, Lees 1980).

1.2 Host range

Historically, European corn borer was a widely distributed insect throughout central and southern Europe, Siberia, northern India, and western China. In the old world it was recognized as a pest of corn, hops, millet, and hemp, but it was not until 1835 when the species was first recorded as an important economic threat to crops (Caffrey and Worthley, 1927). European corn borer is now a serious pest of maize (*Zea mays* L.) and several other crops including peppers, tomatoes, potatoes, snap beans, sorghum, and cotton.

Although ECB is considered primarily a pest of those crops, it has a wide range of host that includes almost any herbaceous plant species with a large, succulent stalk suitable for larval penetration (Hudon and Leroux 1986, Bourguet et al. 2000, Stamps et al. 2007). Lewis (1975) reported 234 species of plant in which *O. nubilalis* can become established. In the absence of abundant corn cultivation in the northern United States and southern Canada *O. nubilalis* has been found in wild grasses (Dicke 1932), broad leaf weeds, potato, *Solanum tuberosum L.*, bean, *Vicia faba* L., and egg plant, *Solanum melongena* L (Hudon and Leroux 1986). *O. nubilalis* adults use grassy areas near the border field as sites for mating and rest (Caffrey and Worthley, 1927; Pedigo, 1989).

1.3 Variation in voltine expression and sex pheromone system:

Genetic types of European corn borer are defined in several ways: (1) sex pheromone communication system (2) voltinism expression, and (3) length of post-diapause development. In Iowa, Sparks (1963) first determined the presence of an *O. nubilalis* sex pheromone, a chemical released by the female moths to attract male moths

for mating. The sex pheromone was a combination of two isomers of 11-tetradecenyl acetate produced by the female (Klun 1968, Klun and Brindley 1970). During the time of its introduction, *O. nubilalis* was apparently polymorphic in respect to the production of either a Z strain, a chemical mixture of 97%(Z) -11-tetradecenyl acetate and 3%(E)-11-tetradecenyl acetate or an E strain 1%(Z)-11-tetradecenyl acetate and 99%(E)-11-tetradecenyl acetate. *O. nubilalis* males are attracted to females that produce the E or Z mixture. In North America, *Z* emitting and responding *O. nubilalis* are common in the southern and central states (Showers et al. 1974, Struble et al. 1987, Kochansky et al. 1975). On the other hand, *E* emitting and responding populations are limited to Quebec, Canada (McLeod et al. 1979) and the northern United States (Carde et al. 1975).

Voltine expression refers by the number of generation produce by a given population in a specific geographic region. The timing of larval diapause controls the emergence of voltine populations in European corn borer. Beck (1960) described diapause as a genetically determined, suspended physiological condition induced by environmental factors such as photoperiod, temperature, and possibly by the nutritional quality of the food. Timing of post-diapause development is determined by the number of heat unit or degree-day required for an over wintered larvae to pupate after the termination of diapause in spring. The number of generational ecotypes in a particular growing season depends largely on the annual degree-days or heat units of the region.

Based on the production of chemical pheromones and voltine expression, Hudon et al (1989) suggested the existence of three races in *Ostrinia nubilalis* species: E-bivoltine, Z- bivoltine, Z- univoltine. In some regions only one race is dominant where as there are some regions of coexistence among the races. Existence of those races in a

region influences the timing of adult emergence, the period of insect's activity, and the pest management of that region.

1.4 Factors influencing diversity and variation in the species:

It is not clear whether a single, multivoltine or both types were introduced in North America; the first observation of the species in the North America was univoltine. Shortly after its discovery, however, *O. nubilalis* demonstrated voltine diversity and it was speculated that at least two voltine types were introduced. Vinal (1917) reported the presence of a bivoltine types in the eastern Massachusetts. In 1919, a univoltine type was discovered near the Lake Erie region and in western New York (Felt 1919). By the late 1930s, a two generation per year type was established in the eastern and north central states. The two generation per year type spread rapidly westward and soon became dominant in the central Corn Belt. The bivoltine population reached Illinois in 1939, Iowa in 1942, and Nebraska in 1946. The univoltine population expanded its range northward into northern Minnesota, North Dakota, and the Canadian provinces of Quebec, Manitoba, and Saskatchewan.

Showers et al. (1975) described three ecotypes: a northern type with a single generation per year or univoltine, a central type with two generations per year or Bivoltine, and a southern type with three to four generations per year or multivoltine in North America. Showers (1979) estimated the degree day requirements to peak pupation (50% pupation) for three European corn borer populations: one collected from the area of the original bivoltine population in Massachusetts (Vinal 1917), the second one collected from the area believed to be a region of univoltine population near Toledo, Ohio (Felt 1919) and a third population from farther west. The populations collected from

Massachusetts (bivoltine) and Ohio (univoltine) suggested a clear difference in the number of degree-day requirements to reach 50% pupation after diapause. Showers found the Ohio population showing univoltine characteristic with less degree-days required (for 50% pupation) than the original univoltine population. However, the reduction in the degree-day requirements might be the result of a genetic shift of the natural population after invasion of a bivoltine population during the time of rapid ECB expansion during 1947-1950 (Showers1981). Showers (1979) reported that the mean number of degree-days required for invading ECB populations of Minnesota was consistent with the univoltine population of western New York. On the other hand, invading populations in Illinois, Iowa, and Kentucky expressed similar degree-day requirements as the invading bivoltine population (post 1943) of Ohio. Interestingly, specimens colleted from near the original introduction site in Massachusetts and from farther south in eastern Virginia and North Carolina had similar degree-day requirements for 50% pupation even though these two regions are 800-1100 miles apart from each other. Based on these results, Shower et al. (1975) suggested the presence of three voltine ecotypes: univoltine, bivoltine, and multivoltine in North America.

Today, both the univoltine and multivoltine ecotypes of European corn borer are recognized across the pest's geographic range, along with two sex pheromone races (Mason et al. 1996). In the central Corn-Belt, bivoltine is the dominant ecotype but in a warmer and longer growing season a third or fourth generation may emerge.

In Central Pennsylvania, both univoltine and multivoltine ecotypes of European corn borer co-exist and the two ecotypes emerge in distinct periods of the growing season (Calvin and Song 1994). The first generation bivoltine flight was was observed in late

May to mid June and the second-generation bivoltine was observed in Mid August. In between the two bivoltine ecotypes, the univoltine ecotype was observed in mid July and overlapped population with the bivoltine flight. In the cooler northern regions of the U.S. and in the Quebec, Canada the univoltine population is dominant.

1.5 ECB biology and development

The life cycle of European corn borer is made up to four life stages: egg, larva, pupa, and adult. Completion of these four stages constitutes a generation. During larval development, *O. nubilalis* completes five instars. During the fifth instar all the healthy larva either prepare to pupate for further development or enter a state of suspended development called diapause. Diapause is a physiological condition triggered in response to day length, temperature, genetic composition, and perhaps by the nutritional quality of its food. Diapause is similar to hibernation of some organisms but differs in that the organism must accumulate a minimum number of cold days before it can resume normal development. In this physiological condition an organism suspends its development to preserve energy during harsh environmental conditions. During midsummer to autumn, shorter day-lengths and lower temperatures trigger diapause induction in *O. nubilalis*. After overwintering, fifth instars resume development towards pupation and emerge as first generation adults.

Diapause in insects can be either obligatory or facultative. Obligatory diapause in organisms is control by genetic characteristics where an organism must enter diapause irrespective of environmental conditions. Facultative diapause is controlled by environmental clues detected by the organisms during a specific stage of life. Based on the environmental cues, an organism can estimate whether there will be enough time

before winter to complete its life cycle or to reach an appropriate life stage to enter diapause. Diapause in *O. nubilalis* is believed to be facultative because the induction of diapause depends upon environmental factors.

First generation multivoltine European corn borers prefer earlier planted cornfields that have reached about 46 cm tall as oviposition sites (Spangler and Calvin 2001). Upon emergence, the moths fly to nearby action sites composed of dense vegetation. The vegetation provides drinking water droplets deposited from dew or rain. Water uptake is very important for the moth's survival and reproduction. Drinking water initiates the release of sex pheromones from females. The microclimate and the emission of pheromones in the action sites facilitate larger aggregation of European corn borer and provide the female moths a better condition for resting and mating. Females usually mate in their second night after emergence and then leave the action site for egg deposition on its preferred host plants. After emergence female moths require a pre-oviposition period of three to five days. In a warm, calm evening a female may lay several egg masses on the basal two thirds of the leaf blade near the midrib on the underside of the corn leaves. After the first days of egg-laying female moths return to the action site for feeding, resting, and sometimes re-mating (about 5% female re-mate).

Depending upon the temperature and weather conditions, eggs hatch with in 3 – 7 days. Adults from the first generation ECB deposit eggs from 260 to 535 DD (in Celsius) with 50% egg deposition being complete by 385 DD (base threshold temperature = 12.5^{o}C) (Calvin 1995). In summer when the average daily temperature is 25^{o}C the first generation egg hatch occurs within 3-5 days. A first generation neonate larva then searches for shelter for protection from predators, parasitoids and extreme weather

condition. Larvae crawl into the developing whorl and feed on young leaf tissues of the unfolded leaves. The first and second instars feed on the mesophyll of leaves leaving the transparent epidermal layer called "windowpane" effect on the leaf surface. During the third and forth instars, when 407 to 736 DD have accumulated since spring pupation, first generation larvae either bore into the stem or feed around the stem. During the summer, in an area of the Z-multivoltine ecotype, the *O. nubilalis* pupates after about 636 to 901 DD have accumulated. Fifty percent pupation happens at 848 DD. The first generation adults emerge between 723 to 998 DD and peak emergence occurrs at around 848 DD. The moth then flies to action sites and then to host plants.

Second generation female moths prefer more succulent, late planted or larger corn plants as their oviposition sites and lay eggs in a bell shape distribution around the ear leaf (Spangler and Calvin 2001). Several researchers found that chemical volatile compounds released by the green silk in the corn plant are used by females to orient their oviposition (Moore 1928, Cantelo and Jacobson 1979). In the absence of a preferred host plant stage, females search for an alternative hosts such as beans, peppers or other suitable host plants. Neonate larvae migrate to the leaf collar area and feed on pollen, husk tissue or developing kernels of the corn plants. Like the first generation, second-generation 3^{rd} – 5^{th} instar larva either bore into the stem or feed around the stem. In their third and fourth instars most of the larvae will bore into the stalk, however, a portion of them still feed within ear, sheath, and collar.

Fifty percent of the second-generation larvae reach fifth instar by 1198 DD. After completion of the fifth instar ECB larvae pupate and eventually produce another moth flight if they do not enter diapause for overwintering. In the middle and upper corn-

growing region of the United States the majority of the fifth instar larva will move in the lower part of the corn stem and enter diapause to overwinter. In the late summer in the southern United States corn growing area, a third and even fourth flight of moths may occur during the long, warm season. In cooler areas where the annual degree-day accumulation is less than 1198 the population is usually dominated by the univoltine ecotype. At any geographic location an additional or partial generation may occur during warmer years. When this occurs, larvae from the terminal generation may develop to the 5^{th} instar and enter diapause. Later developing larvae that do not reach the fifth instar before winter will likely die.

During the winter months, diapausing *O. nubilalis* larvae spend their life as full grown larvae inside plant refuse such as corn stubble, cobs, and weed stalks or in other plant debris. In late winter, ECB larvae break facultative diapause (Beck 1987). When spring temperatures exceed the development threshold for the species, the 5^{th} instars develop toward pupation. In central corn growing areas diapause-breaking temperatures are reached in April or May. The threshold temperature is slightly higher for southern populations and lower for northern populations. Calvin et al. (1991) measured the degree day requirements for each biological event for European corn borer populations collected from Iowa, North Dakota, Missouri, Delaware, and Pennsylvania.

Seasonal degree-day accumulations are calculated for *O. nubilalis* from January 1. The first individual in the first generation of multivoltine populations after post pupates at 75 degree-days, and the last individual pupates at 350 DD. Peak pupation or 50% population pupation was around 200 DD. The pupal period requires an additional 150 DD to begin adult emergence. Degree-days are calculated using the methods

described by Arnold (1959) using 12.5^0C as the base threshold for *O. nubilalis* development (Calvin et al. 1991). Apple (1952) estimated the base threshold temperature for *O. nubilalis* development to be 10^0C, but later Arnold (1960) refined the method, estimating 12^0C as the base threshold temperature. Later Jarvis and Bindley (1965) and McLeod (1981) confirmed Arnolds extended base temperature (12.5^0C). Roth and Derron in 1985 used 12^0C as the developmental threshold for the study of *O. nubilalis* moth activity in Geneva, Switzerland. Based on the location, Beck and Apple (1961) suggested that geographical populations may differ in their base threshold temperature. In this dissertation a base threshold temperature of 12.5^0C was used.

1.6 Crop damage by ECB:

In the United States *O. nubilalis* is responsible for more than $1.85 billion in annual losses (Calvin 1995; Ostlie et al. 1997). Bode and Calvin (1990) estimated a 5.48% yield loss per larva per plant from a first generation *Ostrinia nubilalis* infestation, while the second generation causes only 2.77% yield loss per larva per plant. Although per unit crop loss is less in second-generation infestations, total yield loss is much higher during the second-generation because infestations are larger. The average number of larvae per plant in the second generation is 1.56 compared to 0.37 for the first generation. Nationwide, annual average loss from *O. nubilalis* was estimated to be between 6-7% (Calvin 1995) with average losses in individual fields ranging from 1 to 12% (Patch et al. 1951).

A corn plant goes through a series of phenological stages during its growth and development. Hanway (1966), described nine stages of development for field corn. Field corn cultivation faces yield losses due to the physiological damage from both 1^{st} and 2^{nd}

generation European corn borer. First generation larvae primarily feed on plants in early whorl-stage designated as stage 4 (corn plants with 4 leaves). Huge yield losses can occur if European corn borer infests all stages of corn development especially if damage occurs before ear kernel fill. Corn plants are more susceptible to ECB damage from the 6th-leaf stage (stage 5) through physiological ear maturity. Stalk tunneling during the vegetative stage has much greater impact on the plant growth resulting shorter plant with fewer and smaller leaves. Damaged vascular bundles in the vegetative stage reduce the movement of water, hinder photosynthesis and restrict nutrient flow during kernel filling period. Both Calvin et al. (1988) and Bode and Calvin (1990) found that injury from stalk tunneling decreased from the beginning of reproductive stage to physiological maturity (stage 7-9) thus reducing ear weight loss. The yield loss is very little when stalk tunneling happens late in the period of kernel filling unless there is significant stalk lodging and ear dropping because of shank damage.

Setting a standard economic injury level for European corn borer across the entire corn growing region or across the geographic range is difficult because the synchrony between corn phenology and insect development differs between regions and even fields with different planting dates. Plants infested in their early developmental stages between 10 leaf to mid-whorl are more likely to cause economic losses than those where feeding is nearer to physiological maturity (stage 9). Corn plants infested by a single larva result in 5 and 6 percent grain yield loss during the whorl stage compared to 2 to 4 percent yield loss in plants infested during the ear development stage. Together with prolonged drought and high wind, significant European corn borer tunneling can increase the yield loss per larva per plant up to 12 percent.

Yield loss could be staggering because of the disease incidence due to ECB damage. Studies suggested a close relationship between second and third generation European corn borer infestation and incidence of stalk rot disease caused by two fungi *Fusarium moniliforme* and *Gibberella zeae*.

Infestation from only one larva can damage up to 4 internodes weakening the stalks and ear shanks. Yield loss caused by direct grain feeding is insignificant for field corn but has significant impact on sweet corn, popcorn, and seed corn marketing for aesthetic purpose.

1.7 ECB management:

Intelligent management requires a better understanding of field population level and developmental stages of pest species (Manley 1995). Labatte et al. (1997) estimated the natural mortality of *Ostrinia nubilalis* at 74% mortality in the 1st instar, 13 % in 2nd and 3rd instar and 16% in 4th instar. Before the invention of Bt corn, to control the surviving larvae several management tactics were developed and implemented but none of the technology has shown complete control to prevent significant yield losses. A good management decision process requires a complete understanding of life history parameters of *O. nubilalis* ecotype population.

The following sections will discuss the influence of voltine diversity on some of the management tactics used by the growers.

1.7.1 Cultural practice:

Cultural practices are effective tools to reduce local European corn borer infestations especially in the area of small field cultivation. Methods used by the Pennsylvania corn growers include manipulation of planting dates, row spacing, proper

tillage, destroying post harvest residues, appropriate fertilization, and irrigation (Sked, 2003).

Planting date significantly influences the population dynamics of the *O. nubilalis* (Chiang and Hodson 1963). In one-generation per year areas, early planting with resistant corn hybrids will result in minimum larval feeding, stalk tunneling, and broken stalks because of DIMBOA concentrations in the young leaves. Early planted corn (before 1 May) in central Pennsylvania is more preferable to first generation *O. nubilalis* while second generation larvae could not infest the mature, harder, dry stalks (Huber and Neiswander 1928). However, if the planting date is too late (after June 1) a second generation outbreak occurs because the larva will get their preferred succulent and nutrient rich corn stalks in the late whorl stage (Chiang and Hodson 1963, Hill et al. 1967). Extremely early-planted hybrids may face severe infestation by the first generation larvae if moth emergence occurs during the time of tasseling and silking. Everly (1959) indicated that the peak oviposition period for *O. nubilalis* is when the corn plant in the mid-silking stage. Other than a few ECB tolerant hybrids, corn cultivated in early or late will result in yield losses from broken stalks and ear damage by the second generation infestation. In an area of three or four *O. nubilalis* generations, corn cultivation will be subject to at least two generations of attack. Early planted fields will suffer infestation from first and second generation and the late planted will suffer from the second and third generation.

Tillage practices are effective tools in controlling over-wintering larvae in the corn filed. Plowing just before pupation is an effective tillage practice likely to be based on the regional degree-day accumulation for first generation pupation. Plowing in early

spring and adjusting planting time can reduce significant *O. nubilalis* infestation (Patch 1942, Everly 1959).

1.7.2 Biological control:

Continuous attempts have been made to control *O. nubilalis* using biological agents. During the 1930's several parasitoids were imported into the United States from the native ECB distribution in Asia and Europe (Jones 1927). Baker et al. (1949) listed 23 imported and 29 native parasitoids of *O. nubilalis*. Out of those 23 imported parasitoids only 7 parasitoids established in the U.S. (Hudon et al. 1989). Among the parasitoids released, *Macrocentrus cingulum* Brischke, *Lydela thompsoni* Herting, *Eriborus terebrans* Gravenhorst and *Sympiesis viridula* (Thomson) are the most established species (Coll and Bottrell 1992, Losey et al. 1992)

At least three native parasitoids, *Trichogramma minutum* (Hudon and Lepoux 1986), *Trichogramma brassicae* (Mertz et al. 1995), and *Trichogramma nubilale,* have been used to parasitize 3.6%– 80% *O. nubilalis* eggs (Hudon and Lepoux 1986, Burbutis and Goldstein 1983). A closely related wasp *T. ostriniae* imported from China in 1990 was found to be effective in controlling 97% eggs in the eastern U.S. (Burbutis and Goldstein 1983).

Macrocentrus cingulum is now the most common parasitoids in Massachusetts, Delaware, and Connecticut. It is also reported as important parasitoids in other states such as Maryland, Virginia, West Virginia, New York, Ohio, and North Carolina (Peairs and Lilly 1975, Andreadis 1982, Romig et al. 1985, Mason et al. 1994). Phoofolo et al. (2001) found up to 31% larval infestation by *M. cingulum*.

Four types of entomopathogenic agents such as fungus, microsporidia, bacteria and virus are very common in spreading diseases of *O. nubilalis* (Hudon et al. 1989). The synchrony between the pathogenic agent and the proper life stage of host is very important for effective management. Another pathogen *Nosema pyrausta* is a potential biological agent (Andreadis 1987) but lower survival in the winter limit its effectiveness for the second-generation *O. nubilalis* (Siegel et al. 1987). *Beauvaria bassania* (Balsamo) was reported as one of the major fungal pathogen of overwintering ECB (Hudon et al. 1989, Losey et al. 1992). In control conditions, Phoofolo et al. (2001) found 35 to 84% of *O. nubilalis* larvae infected by *N. pyrausta* and 0 to 21% of larvae infected by *B. bassania*. A naturally occurring bacterium, *Bacillus thuringiensis,* commonly known as *Bt,* is widely used as a biological control agent for many Lepidopteran insects including *O. nubilalis. Rachiplusia ou* a multicapsid nucleopolyhedrovirus (RoMNPV) virus was also successful in infecting *O. nubilalis* (Robert et al. 1999) . Although estimating actual mortality by this pathogen is difficult because it often causes chronic sublethal effects such as decreased fecundity and shortened adult life (Kramer 1959).

Several arthropod predators such as coccinellids, chrysopids, nabids, syrphids, pentatomids, and mirids commonly feed on the eggs of European corn borer. *Coleomegilla maculata* (coleoptera: Coccinellidae) a naturally occurring beetle consumed 6.1% -50% of *O. nubilalis* eggs (Hudon and LeRoux 1986, Phoofolo et al. 2001). In Maryland, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) has been found to be an important larval predator (Coll and Bottrell 1992). Besides arthropod predators several birds are found to be predators of ECB larvae (Coll and Bottrell 1992, Baker et al. 1949).

Other than biotic mortality factors 97% to 99.1% larval mortality were estimated by abiotic factors (Siegel et al. 1987, Ross and Ostlie 1990)

1.7.3 Genetic control:

Genetic control tactics for European corn borer are primarily the development of resistant host plant varieties. Higher concentrations of 2-4-dihydroxy-7-methoxy, 1-4-benzoxaxine-3-one DIMBOA provides only leaf feeding protection from the first generation larval feeding, but no protection from stalk tunneling because in the later stages of corn plant development DIMBOA concentrations are naturally reduced (Klun et al. 1967, Klun and Robinson 1969, Klun et al. 1970).

Because of the continuous infestation in all stages of corn plant, corn breeders took voltine diversity of European corn borer into consideration (Hudon and Chiang 1991). Early research efforts tried to identify resistant corn genotypes based on feeding deterrence, oviposition repellancy, or some sort of physiological fitness tolerating serious injury by the European corn borer (Marston 1930). Since then a number of genotypes have been developed that are resistant to leaf feeding from first generation univoltine and multivoltine ECB (Onukogu et al. 1978, Guthrie 1988, Barry and Darrah 1991, Scott et al. 1996, Krakowsky et al. 2007). Today almost all commercially available corn hybrids have moderate to high level of resistance to both first and second generation leaf feeding.

1.7.4 Chemical control:

Appling chemical control tactics for controlling all genetic types (pheromone races) and generations (ecotypes) of European corn borer are effective as a component of Integrated Pest Management (IPM). Chemical control methods are used in combination

with other control tactics when the *O. nubilalis* population exceeds the economic threshold level. Chemical control for ECB is only effective for first and second instar larvae since later stages are not susceptible (Crawford 1924). Since, chemical control is effective only on certain life stages of ECB, identifying appropriate stages, determining application timing and the expected level of control is the key to gain benefit. Chemical control largely depends on the geographic location, topography, farm size, and pest phenology (Tollefson and Calvin, 1994). Temperature is a key climatic component influencing the rate of ECB development. In cooler regions, the development will be much slower and generation time will be much longer then in warmers areas. The relationship between the development rate and time length determine the span of the susceptible life stage.

1.7.5 Transgenic Control:

Commercial use of transgenic corn (*Zea mays* L.) expressing the delta endotoxin produced by *Bacillus thuringiensis* Berliner to control *O. nubilalis* began in 1996 (Koziel et al. 1993, Fischhoff 1993). The popularity of Bt corn increased in the United Sates and around the world. Bt corn's season long, persistent control of ECB coupled with the widespread resistance in some lepidopteron pest populations against conventional insecticides made it a popular pest management tactic (Tabashnik 1994, Rice and Ostlie 1997, Pilcher et al. 2002). According to the national corn growers association, in 2005, 45 percent of field corn planted nationally contained a *Bt* gene in comparison to 4% in 1996.

Concerns have been raised about the long term viability of Bt corn because some insects have already developed resistance to commercial formulations of folarly-applied

Bacillus thuringiensis (Mcgaughey 1985, Gould 1998, Mcgaughey and Johnson 1992, Mcgaughey and Whalon 1992, Shelton et al. 1993, Whalon et al. 1993, Tabashnik 1994, Huang et al. 1997, Wolfenbarger and Phifer 2000, Wolfenbarger et al. 2002, Reardon et al. 2004,). Resistance to Bt toxin has already been detected in the field population of diamondback moth, *Plutella xylostella* L. (Kirsch and Schmutterer 1988, Tabashnik et al. 1990, Tabashnik 1994, Kao et al. 1994). In addition, selection studies of laboratory populations of several insect species expressed resistance to Bt toxin (Schnepf et al. 1998, Frutos et al. 1999, Sanchis 2000, Van Rie 2002). ECB collected from northeast Kansas and Iowa (Huang et al, 1997, 1999a) were found to be resistant to commercial formulations of *Bt*. Increased resistance to Cry1Ac and Cry1Ab *Bt* toxin was also documented in a population collected from Southeastern Minnesota (Bolin et al. 1999). Low levels of resistance to Cry1Ab were determined in lab populations of ECB in Nebraska (USA) and in Italy (Chaufaux et al. 2001). The number of generations produced per year by a specific pest population can influence the rate of resistance evolution; more generations per growing season would generate more opportunities for the selection of a resistant genotype.

1.8 Synthesis of current knowledge and research questions:

Since its introduction to North America *O. nubilalis* has shown voltine diversity. Early research focused on range expansion of the pest facilitated by environmental influences such as photoperiodic threshold, latitude, temperature and length of growing season. Few studies addressed the genetic basis of voltine expression in North America. Smith (1991) & Thorpe (1995) speculated that hybrid corn lead to a “host-race”

formation. Bush (1969) hypothesized that sympatric speciation can occur in the absence of geographical separation by a host shift which causes reproductive isolation. A recent study by Malausa, et al. (2005) showed that *O. nubilalis* feeding on different host plants in the same geographic location can show a significant level of reproductive isolation. This phenomenon could explain variable voltine expression in *O. nubilalis* in relation to the host plant phenology where multivoltine and univoltine feed on different stage of corn plant and emerge in distinct period.

In the 1970s McLeod et al (1979) found the occurrence of bivoltine ECB populations in Quebec, Canada and thought it was the result of a recent invasion from New York and ruled out the possible genetic shift from the long established (Hudon 1959) original univoltine population.

A European corn borer population in central and western New York has been known to be a univoltine population since 1916 (Felt, 1919). In 1960, a bivoltine population was seemingly predominant in these areas (McEwen and Kawanish.Cy 1967, McEwen et al. 1968). Early researchers thought that it was a recent voltinism shift from a single generation to a multi generations per year population. However, a black light trap catch study by (Eckenrode et al. 1983) and a sex pheromone blend analysis study by Roelofs et al. (1985) suggested the existence of distinct bivoltine *E*, bivoltine *Z*, and univoltine *Z* types of European corn borer populations in these areas. Although there might be some interbreeding between univoltine and bivoltine population, their studies (McEwen et al 1968, Eckenrode et al.1983, and Roelofs et al. 1985) suggested that each voltine population maintained its integrity and expressed very little genetic shift of voltinism and sex pheromone blend. Based on the findings (Chiang 1972, McLoed 1976,

Palmer et al. 1985, and Lee et al. 198), Showers suggested that the establishment of bivoltine populations in the historical univoltine areas of Minnesota, North Dakota, and the prairies of Canada is due to abundant planting of hybrid corn and their longer succulent stage through August. Warmer season and long lasting succulent corn plants serve as a reservoir for the wind-borne bivoltine European corn borer population. Showers also supported the earlier findings (McLeod, 1976, McLeod et al. 1979, Eckenrode et al. 1983) that in a sympatric region because of the asynchrony between univoltine and bivoltine populations, univoltine will remain a distinct ecotype even as an unnoticed entity.

To understand the complete population dynamics of the ECB ecotypes study of the other life history parameters of univoltine and multivoltine populations is essential. The present study was undertaken to compare life history parameters, *Bt* toxin susceptibilities, parasitoid impact and ecotype patterns of *O. nubilalis*. These studies will contribute to further understanding the adaptation and evolution of ecotype populations of an introduced insect species.

This dissertation has seven chapters; the first chapter is the introduction and an overview of *O. nubilalis*. The second chapter is a comparison of life history parameters between temporally and spatially isolated univoltine and multivoltine *O. nubilalis* populations of Pennsylvania. The third chapter is a comparison of reproductive parameters between univoltine and multivoltine ecotypes. The fourth chapter describes the susceptibility of univoltine and multivoltine *O. nubilalis* population to Cry1Ab and Cry1F *Bt* toxin. The fifth chapter details the influence of a major parasitoid *Macrocentrus cingulum* on the abundance of univoltine and multivoltine population. The sixth chapter

explains the role of seasonal degree-day accumulation on the patterns of univoltine and multivoltine ecotype population and the seventh chapter is an overall summary and conclusion.

1.9 References:

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Chapter 2.

Life history comparison between univoltine and multivoltine *Ostrinia nubilalis* ecotypes that exist in temporally separated and spatially isolated populations in Pennsylvania, USA.

2.1 Introduction:

The European corn borer (ECB), *Ostrinia nubilalis* (Hubner) was first noticed in North America in 1914 infesting 100-square miles of sweet corn near Boston, Massachusetts (Vinal 1917). Early observation suggested the insect displayed bivoltinism with the maturing larva of the second generation entering diapause for overwintering. A few years later, in 1919, a univoltine ECB population was noticed west of the Lake Erie region in New York State (Felt 1921). The two *O. nubilalis* populations (Massachusetts and New York), separated by only a few hundred miles displayed differences in spring pupation periods, adult emergence, and voltinism (Barber 1925). Caffrey and Worthley (1927) reported that progeny of ECB larva collected from New York and reared in cages in Massachusetts, expressed univoltinism, while the Massachusets population expressed bivoltinism. This was the first evidence of voltinism differences in North America.

Later, Showers et al. (1975) described three ecotypes, a northern type or single generation, a central type or two generations and a southern type or three to four generations per year type. Today, both univoltine and multivoltine ecotypes of European corn borer are recognized across the pest's geographic range, along with two sex pheromone races (Mason et al. 1996). In Central Pennsylvania, both univoltine and multivoltine ecotypes of European corn borer co-exist and the two ecotypes emerge during distinct periods of the growing season (Calvin and Song 1994).

Although, *O. nubilalis* are native to Europe, both in Europe and in North America the insect exhibits two behaviorally isolated strains that differ by the female sex pheromone blends and response by the males. The Z strains females produce a 97:3 (Z)- and (E)- 11 tetradecenyl acetate, where as E strains females produce a 1:99 (Z)- and (E)- 11 tetradecenyl acetate (Klun et al. 1973). Beside the pheromone communication system ECB populations are also characterized by differences in the number of generation they produced per year.

European corn borer voltinism is dependant on the post diapause developmental (PDD) time (i.e. thermal unit required by an individual over-wintered larva to pupate under favorable temperature) and photoperiodic conditions. In the northeastern United State, there are regions where ECB are bivoltine and areas where ECB are univoltine (Roelofs et al. 1985).

In most regions of the northeastern U.S. univoltine and bivoltine populations are geographically separated. However, there are some areas where the two populations co-exist. In these areas differences in post-diapause developmental degree-day requirement resulting a temporal isolation. Bivoltine populations have a flight peak in June and another flight peak in August. Between these two flight peaks, the univoltine flight peak is observed in mid-July. Although, patterns of mating isolation are established by geographical isolation, hybridization and gene flow can occur between the two voltine populations (Dopman et al. 2005), particularly when they are sympatric.

Variable adult emergence time influence the life history characteristics such as offspring growth and development, reproductive success, evolution, sexual behavior, population biology, and sex ratio of an insect species (Seger 1983, Evans and O'Neill

1988, Richards 2001, Brockmann 2004). Variation in adult emergence time among individuals within a species can also affect the intensity of resource allocation, sexual relations and interaction with parasitoid populations (Waldbauer 1978, Hastings 1989, Molumby 1995). Although, univoltine and multivoltine populations exist sympatrically in central Pennsylvania, the timing of the adult peak emergence period makes them partially temporally isolated.

Although, the existence of co-occurring voltine or ecotype population has been known for some time, very little is known about the developmental patterns of the univoltine and multivoltine ecotypes when they occur sympatrically. The objective of this study was to compare life history characteristics of co-occurring Z- univoltine and Z-multivoltine *O. nubilalis* population in central Pennsylvania. Thus studies were design to address the following questions: (1) Does *O. nubilalis* post-diapause development pattern (degree-day accumulation) varied between univoltine and multivoltine populations collected from a sympatric region. (2) Does the development pattern varied among spatially isolated voltine populations. (3) Does the development pattern of non-diapause univoltine and multivoltine larvae of these ecotypes over consecutive generations (F_1-F_3) follow the pattern of the parent population collected from the field. Post-diapause development pattern of co-occurring voltine population from three addition locations in Pennsylvania were also observed in 2005.

2.2 Materials and Methods

2.2.1 General procedure:

Colony Collection:

European corn borers, *Ostrinia nubilalis*, were collected as over wintering larvae from corn stubble in fields from several locations in Pennsylvania, USA. During the study period (2002-2005), between mid-April to early-May, approximately 127- 450 diapausing 5^{th}-instar larvae were collected from the Russell E. Larsen research center (40^{o} 42` N $77^{o}$ 57` W and elevation 1996ft) - a region of temporally separated sympatric voltine populations near Rock Springs, Pennsylvania. In 2005, overwintered larvae were collected from an additional three locations: Landisville (SE Pennsylvania), Erie (NW Pennsylvania), and Bradford (North of Pennsylvania). These populations were transferred to the laboratory and reared in environmentally controlled growth chambers.

Field collected larvae were individually placed into a 30 ml plastic cup, and then transferred to the Department of Entomology at the Pennsylvania State University. A water soaked small cotton ball was placed in each cup to provide sufficient moisture and prevent desiccation and entrance to diapause (Babcock 1924, 1927, Mellanby 1958, Beck 1967). The cotton ball was rewetted at two days intervals until the adults emerged. During diapause termination water is essential for re-establishment of optimum tissue water balance and activation of endocrine functions to expedite metamorphosis (Beck and Hanec 1960, Beck 1967). After preparation, cups were kept in an environmental growth chamber (Revco model no. RI-23-555-A) at 25^{0}C ($\pm$ 0.5^{0}C) and 18L: 6D photoperiod. No diet was provided during the post-diapause development period. Larvae in each cup were marked with a serial number, and the date of collection was noted to

calculate the exact number of degree-days that a larva experienced while in the growth chamber. To avoid contamination aseptic conditions were maintained for the entire procedure from field collection to adult oviposition. Sterile forceps were used to transfer a larva into the cups. Environmental growth chambers, trays, and the rearing cages were cleaned and disinfected periodically with a 1% bleach solution.

Larvae were examined daily for pupation, parasitoid infestation, and death caused by disease or injury during the collection process. The fates of field-collected larvae were explained using the categories described by Losey et al. (1992). Larvae exposed to any unusual circumstances such as disease, injury, or parasitoids were isolated into different trays to avoid contamination. When an individual European corn borer was found pupated, the date of pupation was recorded. After pupation, a pupa was left in the growth chamber until it emerged as an adult, and the date of emergence was recorded.

Colony establishment:

When collected in the field, it is not possible to determine if a larva is univoltine or multivoltine. However, previous work (Calvin and Song 1994) showed that univoltine *O. nubilalis* required between 351 to 650 plus degree-days to reach pupation in the spring, while multivoltine individuals required between 50 to 350 degree days. For this experiment degree-days were used as a method to separate the voltine types. Degree days were calculated using the methods described by Arnold (1959), using 12.5^{0}C as the base threshold of *O. nubilalis* development (Calvin et al. 1991).

To calculate the degree-days that accumulated in the field prior to the date a larva was collected, temperature data were spatially interpolated to a 1 km^2 resolution for the

geographic coordinates where they were collected (Russo et al. 1987). Any positive degree-days were calculated as accumulated degree-days for a 24-hour period until pupation and adult emergence. The total number of accumulated degree-days experienced by a larva was calculated by adding the number of degree-days accumulated from January 1 to the date a larva was collected from the field to the number of degree-days accumulated after collection and in the growth chamber. Based on 75-350 DD range suggested for the multivoltine spring pupation period and 351-650 DD range for the univoltine spring pupation period (post diapause developments), *O. nubilalis* pupae were segregated into a univoltine and multivoltine population (Calvin and Song, 1994). Since, 350 degree-day is the upper range for multivoltine population and there is some overlap of univoltine and multivoltine pupation periods a 150 degree-day buffer between multivoltine and univoltine post-diapause population was created to minimize misclassification of individual larvae into voltine category. For the muivoltine individuals pupating between 50 -300 degree-days were used to establish a colony. The univoltine colony was established from individuals that pupated after 451 degree-days.

Colony maintenance:

Univoltine and mulitvoltine adults from colonies established using field-collected larvae were transferred into mating cages. Several mating cages were used to maintain and segregate univoltine and multivoltine colonies. To avoid over crowding no more than fifty males and fifty females were placed into a single mating cage.

The mating cage was a one cubic feet six-sided wooden box. Five sides of the wooden box were covered with 256-mesh size aluminum screen and the upper side of the

mating case was covered with a 64-mesh size screen that facilitated oviposition onto wax paper. Cages with adults were placed in a walk-in environmental chamber maintained at 25^0C, and 18:6 (L: D) photoperiods. Relative humidity inside the chamber was maintained above 95% using a volume-controlled humidifier (Vicks vaporizer, model 150) to ensure oviposition. Periodical measurement of humidity was conducted using a digital humidity meter (Labcraft brand model 264-767). A 15% honey solution (15% by volume honey in distilled water) was added as the nutrition source for the adults to maximize their fecundity and longevity. Wax paper was placed on top of each mating cage as a substrate for oviposition. Eggs deposited on wax paper were collected at two to three days intervals depending upon the number of egg masses deposited. Collected egg masses were then place in a screen-topped Mason jar until larval emergence. A moist paper towel was placed at the bottom of the Mason jar to prevent desiccation of the egg masses. The majority of the eggs hatched within two to three days at 25^0C.

2.2.2 ECB development studies

Temporally isolated post-diapause population development:

Post-diapause development patterns of over-wintered *O. nubilalis* larvae collected from corn stubble at Rock Spring, Pennsylvania were investigated during 2002, 2003, 2004 and 2005. Field collected larvae were kept in an Environmental chamber at 25^0C, 18:6 L:D, and ≥ 65% humidity. In 2002, approximately 127 field collected over wintered 5^{th} instars *O. nubilalis* larva were monitored, of which eighty two larvae were pupated. The other forty-five larvae died from causes such as parasitism, a pathogenic disease or an unknown injury. In 2003, approximately 448 over-wintered larvae were collected from

the same location in central Pennsylvania. Three hundred and fifty two larvae pupated and ninety six larvae died. In spring 2004, another 359 over-wintered larva were collected again from the same location. Two hundred and fifty three larvae were pupated and 106 larvae died. In 2005, four hundred and twenty two over-wintered larvae were collected. Two hundred and forty two larvae were pupated and 180 died.

After placement in the environmental chamber, larvae were examined daily for pupation following the procedure described in the colony establishment and rearing section. Date of pupation was recorded and the degree-day requirement for each individual *O. nubilalis* to reach pupation was calculated. Pupae were tracked through adult emergence. The date of adult emergence and associated degree-day requirements were recorded for each of the field collected larvae. Data were then entered into an Excel™ spreadsheet for mathematical calculations and statistical analysis.

Spatially isolated post-diapause populations development:

In 2005, post-diapause *O. nubilalis* development (DDA) patterns were studied for larvae collected at three additional locations in Pennsylvania. One hundred and seventeen over-wintered larva were collected from Landisville (South-east Pennsylvania, 40°12`N 76°46`W and 360ft elevation) a region of predominantly multivoltine individuals. Another seventy-five larva were collected from Erie county, an area (Northwest Pennsylvania, 40°01`N 80°12`W and 837ft elevation) suspected to be dominated by the univoltine population. Eighty-five over-wintered larvae were also collected from Bradford county (North of Pennsylvania, 41°48`N 76°28`W and 759ft elevation), which

is predominantly univoltine. The same procedures used in the temporally isolated populations study were used in this spatially isolated populations study.

Non-diapause (F1) population development:

Two hundred and fifty first generation (F_1); 24-h old larvae, from each of the univoltine and multivoltine populations were transferred into individual diet cups containing approximately 6 mg of diet in each cup. The diet was prepared by adding 162gms of ready mixed artificial diet (*O. nubilalis* diet, Southland Products, Lake Village, AR) into 1 liter of boiling water. Diet cups containing a single neonate larvae were then transferred into the environmental growth chamber. The environmental condition of the chamber were also maintained at 25^0C ($\pm 0.5^0$C), 18L: 6D photoperiod and 65% relative humidity. Each larva was examined daily for pupation. Once pupation occurred, the date was recorded. About 95% of the larvae pupated outside the diet block, so pupation was easily detected. The remaining 5% pupated inside the diet block. To confirm the exact pupation date for these larvae a small insertion was made in the diet block without disturbing the larvae. These cups were marked to do the same procedure until the larvae were pupated or came out from the diet block. This procedure ensures the exact pupation data for each larva. After pupation each individual was monitored until adult emergence. The date of adult emergence was then recorded.

Degree-days were calculated as described in the temporally and spatially isolated study. The procedure was followed on the F_1 to F_3 generations for 2002 and 2003. Degree-day accumulations for univoltine and multivoltine by generations were tabulated (table 2.2 and 2.3) and mean pupation periods were compared using a Two sample T-test

(Minitab 2000) (figure 14 and 15). Data were then entered into an Excel^{TM} spreadsheet for mathematical calculations and statistical analysis. For all the generations and years, the cumulative proportion of pupation and the degree-day accumulation from egg hatch to pupation were plotted.

2.2.3 Model construction:

Mathematical models were constructed by using a logistic function to develop a relationship between seasonal degree-day requirements and proportion of multivoltine and univoltine *O. nubilalis* pupation. The dependent variable in each equation was total seasonal degree days accumulated from day one of the year (1 January) and the dependent variable was the proportional completion of *O. nubilalis* populations entered in to pupation or adult eclosion.

Degree days to pupation were converted to the proportion of individual pupated by a given number of degree-days. These data were designated as observed data. Logistic models were then fitted to these cumulative distributions of observed data. To fit these curves, observed data were then mathematically transformed by the equation y = ln(1/observed value – 1) where, y = transformed value of the proportion larva entered into pupal stage and ln = natural log. A regression analysis was conducted between accumulated degree-days and the observed value and a trend line was fitted to the transformed value to estimate slope, intercept, and R-square values for the logistic equation. If the R-square value were below 0.80, a logarithmic regression transformation procedure was used in the equation to fit the transformed data in an attempt to get a better fit to the data. In this case the slope and intercept parameters were inserted into the equation (1)

$$\ln(1/\text{observed value} - 1) = \alpha + \beta \ln * \text{accumulated degree days} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

Using this model a fitted line was added with the observed value to calculate the number of degree-days required for each phenological event such as multivoltine and univoltine pupation, pupation starting and ending period, peak pupation period, and the proportion of multivoltine and univoltine population for each of the four years and locations (figure 2.1 and 2.2). In this experiment, all the observed data were fitted well, so we used observed values for all estimates reported in table 2.1. A trend of proportional change was also observed during the investigated years. Observed degree-day accumulation pattern and proportion of pupation for the investigated years and locations were reported in table 2.1.

2.3 Results:

2.3.1 ECB development studies results

Temporally isolated post-diapause population development:

Figure 2.1 show the four years of cumulative percentage entrance into the pupal stage of post-diapause *O. nubilalis* larva collected from fields in Central PA. Across all the four years post-diapause larvae started pupation at around 48 – 79 degree-days, with most individuals pupated by 650 DD. At least one individuals required up to 1360.7 degree-days to pupate.

The proportion of post-diapause pupation ≤ 350 DD (segregation points between multivoltine and univoltine ecotypes) varied between years (table 2.1, figure 2.1). In 2002, 58% of the population was categorized as multivoltine and 42 % was categorized as univoltine. In 2003, the proportion of multivoltine (60.22%) and univoltine (39.78%)

was similar to 2002. In 2004 and 2005, however, the proportion of multivoltine decreased to 41% and 38% respectively, and the proportion of univoltine increased to 59% and 62% respectively. The number of degree-days to 50% pupation in 2004 and 2005 consequently increased when compare to 2002 and 2003 data. In 2002 and 2003, 50% pupation occurred at 296.0 DD and 300 DD respectively; where as, in 2004 and 2005, 50% pupation occurred at 403.0 DD and 425.0 DD. In 2002, 77 of 127 larvae were pupated with the last 5% of pupation occurring between 604.9 – 696.8 DD. In 2003, 352 of 448 larva pupated with the last 5% of the larva pupating between 597 – 1360 degree-days. In 2004, 253 of the 359 larvae pupated with the last 5% of the larvae pupating between 708.5 – 1200 degree-days. In 2005, 242 of 422 larvae pupated with the last 5% pupating between 648 – 1239 degree-days. It was observed that 95% of the pupation occurred between 50 to 700 degree days but the rest of the pupation (5%) occurred within a large degree-day regime (700 – 1400 DD) (table 2.1).

Spatially isolated post-diapause populations development:

Figure 2.2 showed the 2005 cumulative percentage entrance into the pupal stage of the post-diapause larva collected from three spatially separated univoltine and multivoltine regions across Pennsylvania. In the Landisville area, a region dominated by multivoltine individuals, post-diapause pupation started at 18.33 DD and ended at 405.0 DD accumulations. In the Landisville 95% of the larvae pupated within 350.0 DD. The remaining pupated between 351 – 405 DD.

In the Erie and Bradford area, the regions of univoltine dominant population, post-diapause pupation started at 75 DD accumulations and ended at 775.0 and 750.0

DD, respectively. Of the larvae collected near Erie only 33% pupated within 350 DD and the rest (67%) pupated between 351-775 DD. Although, the Bradford county region was considered a univoltine region, a higher proportion (76%) of larvae pupated within 350.0 DD compared to those pupated between 351 to 750 DD (24%) (table 2.1). The Landisville colony reaches 50% pupation at 218.3 DD, where the Erie colony reached 50% pupation in 400.0 DD. The Bradford colony reached peak pupation at 237.5 DD. It required 350.0 DD, 625.0 DD, and 462.5 DD for the Landisville, Erie, and Bradford colonies, respectively, to reach 95% pupation (table 2.1).

Non-diapause (F1) populations development:

Multi-generation (F_1-F_3) non-diapause development studies of univoltine and multivoltine colonies were conducted in 2002 and 2003. A summary of the results and statistics for each generation and year are shown in tables 2.2 and 2.3, respectively, for 2002 and 2003.

The cumulative pupation curve for the 2002 F_1, F_2, and F_3 univoltine and multivoltine ecotypes are given in figure 2.3-2.8. In a non-diapause condition, univoltine F_1 larvae started pupation at 237.5 DD and ended at 962.5 DD, whereas multivoltine F_1 larvae started pupation at 212.5 DD and ended at 362.5 DD. The peak pupation period for the multivoltine population (262.5 DD) was lower than the peak pupation period for the univoltine population (287.5 DD). The mean pupation period of univoltine larvae (325.5 DD) was significantly higher than the mean pupation period of the multivoltine larvae (272.2 DD) ($F = 20.54$, $p = 0.0001$, $df = 346$). In the F_2 generation univoltine population started pupation at 212.5 DD and ended at 700.0 DD. The multivoltine F_2 population

started pupation at 218.5 DD and ended at 387.5 DD. The peak pupation period for the univoltine population was 287.5 DD and for the multivoltine population was 258.0 DD. The mean pupation period for univoltine ecotype (314.0 DD) was again significantly higher than the mean pupation period for multivoltine population (257.4 DD) ($F = 39.76$, $p = 0.0001$, $df = 353$). In the F_3 generation, the univoltine population started pupation at 212.5 DD and ended at 562.5 DD, whereas the multivoltine population started pupation at 212.5 DD and ended at 462.5 DD. The peak pupation period was 287.5 DD and 262.5 DD for univoltine and multivoltine populations, respectively. The mean pupation period of univoltine (289.7 DD) was also significantly higher than the mean pupation period of the multivoltine population (268.7 DD) ($F = 21.45$, $p = 0.0001$, $df = 440$). When the degree-days were plotted against the cumulative proportion of pupation, in three consecutive generation univoltine populations required a wide range of degree-day accumulation to become pupae compared to the multivoltine populations (figure 2.3-2.5).

In 2003 when we repeated the experiment with a larger field collected parent population similar results were observed. In 2003, the F_1 univoltine larvae started pupation at 250.0 DD and ended at 1612.5 DD. The multivoltine larva started pupation at 212.5 DD and ended at 462.5 DD. The peak pupation period for the univoltine population was higher (312.5 DD) than the peak pupation of multivoltine pupation (262.5 DD). The mean pupation period (352.4 DD) for the 2003 univoltine F_1 population was significantly higher than the mean pupation period of the 2003 F_1 multivoltine population (268.1DD) ($F = 49.48$, $p = 0.0001$, $df = 380$). The F_2 univoltine population started pupation at 237.5 DD and the multivoltine pupation started at 212.5 DD. In F_2 generation, the univoltine population completed pupation at 1050.0 DD and the multivoltine pupation period

completed pupation at 387.5 DD. The mean pupation period for univoltine (332.3 DD) was significantly higher than the mean pupation period for multivoltine (269.8) ($F = 69.86$, $p = 0.0001$, $df = 380$). The F3 univoltine population started pupation at 250.0 DD and completed pupation at 987.5. The multivoltine pupation started at 225.0 DD and completed at 675.0 DD. The peak pupation period was 300.0 DD and 262.5 DD respectively, for univoltine and multivoltine populations. The mean pupation period for univoltine (322.1 DD) was significantly higher than the mean pupation period for multivoltine (267.6 DD) ($F = 62.15$, $p = 0.0001$, $df = 451$)

Similar development pattern (degree-day range) was observed in each of the three consecutive (F_1-F_3) generations of 2002 and 2003 non-diapause study. Like their parent population univoltine ecotype required a wide range of degree-day accumulation (237.5 to as high as 1612.50 DD) whereas, multivoltine ecotype required a narrow range of degree-day accumulation (212.5 to as high as 462.5 DD) for pupation (Figure 2.6-2.8).

2.4 Discussions:

No previous study has reported in detail the developmental differences between univoltine and multivoltine populations of *O. nubilalis.*. Significant differences were found in post-diapause and non-diapause developmental degree day requirements of univoltine and multivoltine populations and variation in the proportion of voltine types present at a geographic variation between years.

Post-diapause spring pupation started at the same time and ended at similar times across the years. Although, in 2002 pupation ended in 696.8 DD, a larger sample collection in 2003-2005 confirmed that a small portion of a post-diapause *O. nubilalis* population can pupate at up to 1360.0 DD from the start of pupation in early spring. From

the above results, it may speculated that in a longer growing season and/or in a warm summer, a higher proportion of post-diapause *O. nubilalis* larvae will pupate and complete their life cycle to produce a second generation or diapause to contribute to next year populations. The post-diapause univoltine and multivoltine proportion varied between years. The peak pupation period (50% pupation) also varied between years. Historical, environmental conditions such as temperature and photoperiod may influence the ecotype ratio of the next year's ECB population.

Variation in non-diapause development patterns between univoltine and multivoltine ecotypes suggested the presence of a genetic basis for higher degree-day requirements of the univoltine *O. nubilalis* population. Under some environmental conditions, the post-diapause individuals requiring higher degree days before pupate may contribute more of the univoltine genotype to the over all ECB population thus increasing the proportion of univoltine ecotypes. Moreover, our study (chapter 5) found that a differential parasitism rate (between univoltine and multivoltine) by *Macrocentrus cingulum*, the most effective larval parasitoid for *O. nubilalis*, may contribute to the proportional variation in the ecotype population.

In the non-diapause F_1, F_2 and F_3 generations in both years (2002 and 2003) we observed a decrease in higher degree day requiring univoltine larvae and a resulting decrease of skewness in the *O. nubilalis* population curve (figure 2.3-2.8). In the 2002 F_1 population, the last 5% of the univoltine pupation was limited to 512.5 DD to 1612 DD, in the F_2 population the last 5% pupation was limited to 512.5 DD to 1050.0 DD and in

the F_3 generation last 5% of univoltine pupation was limited to 450.0 DD to 987.5 DD. Similarly, in the 2003 F_1 population, the last 5% of univoltine pupation was limited to 512.5 DD to 962.5 DD, in the F_2 generation the last 5% pupation was limited to 637 DD to 700.0 DD and in the F_3 generation last 5% of the univoltine pupation was limited to 412.5 DD to 562.5 DD (table 2.2 and 2.3).

On the other hand, in 2002 and 2003 multivoltine populations expressed little decrease in the number of high degree-day requiring larvae and the population growth curve skewness was not changed in the consecutive three generations. In 2002 F_1 population, the last 5% of the multivoltine pupation was limited to 337.5 DD to 462.5 DD, in the F_2 population 325.5 DD to 387.5 DD and in the F_3 generation last 5% of the univoltine pupation was limited to 325.5 DD to 675.0 DD. Similarly, in the 2003 F_1 generation the last 5% of the multivoltine pupation was limited to 337.5 DD to 362.5 DD, in the F_2 population 288.0 DD to 378.0 DD and in the F_3 generation last 5% of the multivoltine pupation was limited to 325.0 DD to 462.5 DD (table 2.2-2.3). The lower number of higher degree day requiring individuals in the consecutive univoltine generation may be due to genetic drift or founder effect because of the absence of viable mating partners for the later emerged adults. In this way ECB may lose high degree day requiring univoltine genes in consecutive generations (F_1-F_3). On the other hand, multivoltine ecotypes produce a larger number of adults in narrow range of degree-days thus having a greater success in the random mating and maintaining multivoltine genes in the consecutive generations (F_1-F_3). These phenomena strengthen our assertion that *O. nubilalis* field populations experiencing a longer growing season and/or a warmer summer may contribute to a larger population of high degree-day requiring univoltine

adults emerging in the late summer and ensuring that their progeny experience sufficient degree-days to develop to the 5^{th} instar before winter.

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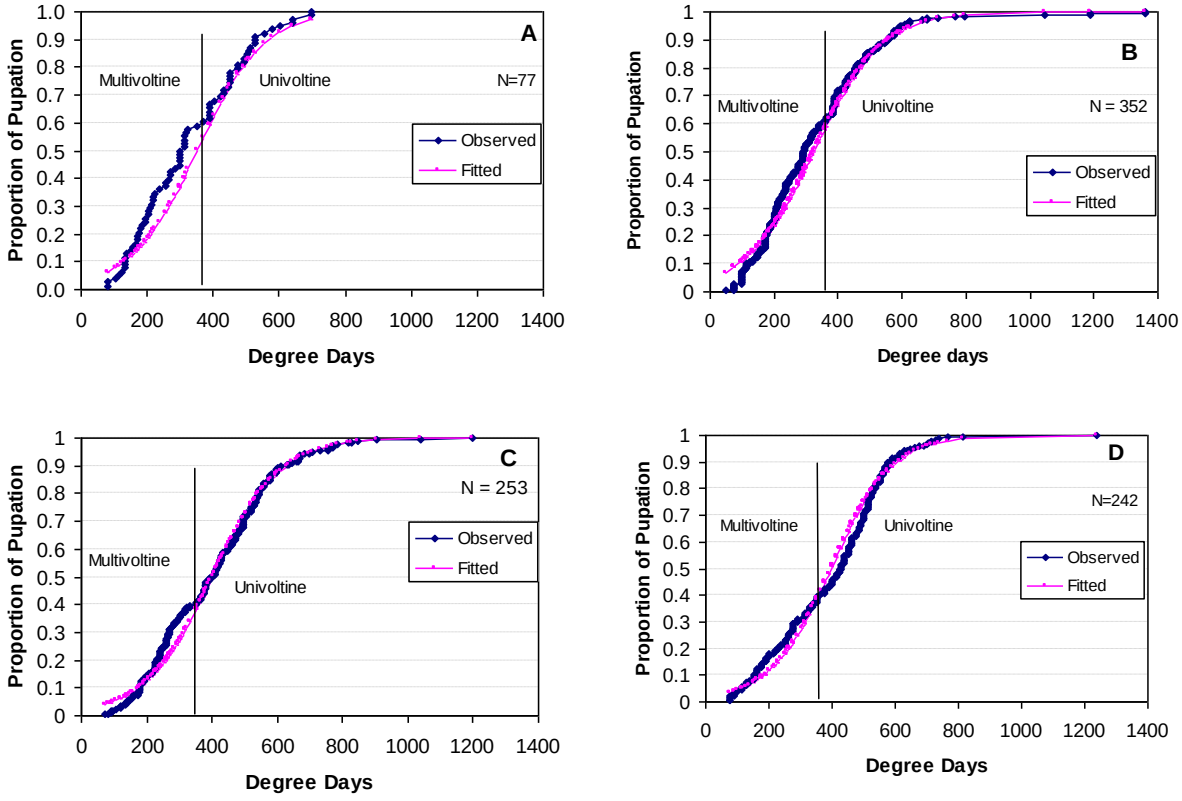
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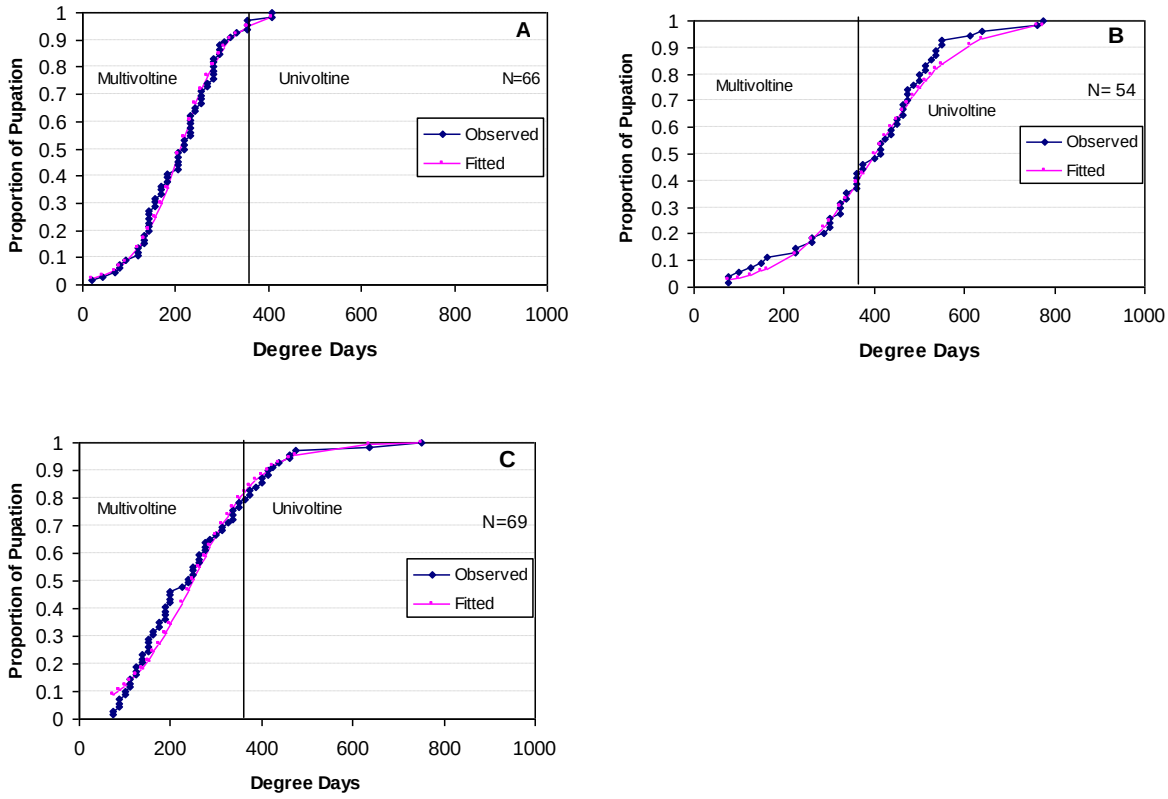
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Figure 2.1: Post-diapause degree-day requirements for pupation of spring field collected *O. nubilalis* larvae using a 12.5°C base threshold temperature when reared at 25.0°C and a 18:6 L:D photoperiod. (A) 2002 (B) 2003 (C) 2004, and (D) 2005 Rock spring, PA population.



Note: Vertical line on 350 DD on the degree days axis is the segregation point between multivoltine and univoltine ecotypes.

Figure 2.2: Post-diapause degree-day requirements for pupation of spring 2005 field collected *O. nubilalis* larvae using a 12.5°C base threshold temperature when reared at 25.0°C and a 18:6 L:D photoperiod. (A) Landisville, PA (B) Erie, PA (C) Bradford, PA



Note: Vertical line on 350 DD on the degree days axis is the segregation point between multivoltine and univoltine ecotypes.

Figure 2.3: Prediction curve for degree-day requirements of non-diapausing 2002 F1 generation of univoltine and multivoltine *O. nubilalis* populations development from egg hatch to pupation at 25^0C and 18:6 (L:D) photoperiod.

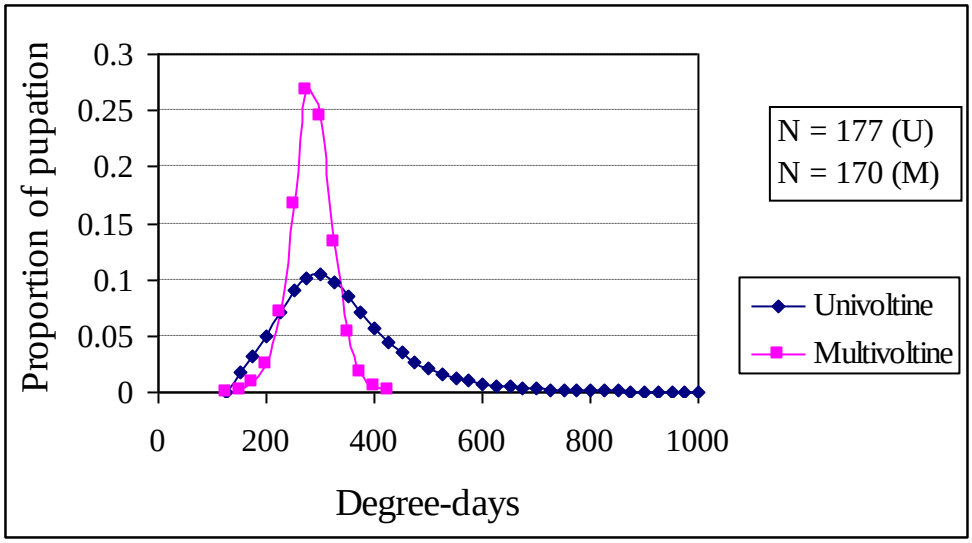


Figure 2.4: Prediction curve for degree-day requirements of non-diapausing 2002 F2 generation of univoltine and multivoltine *O. nubilalis* populations development from egg hatch to pupation at 25^0C and 18:6 (L:D) photoperiod.

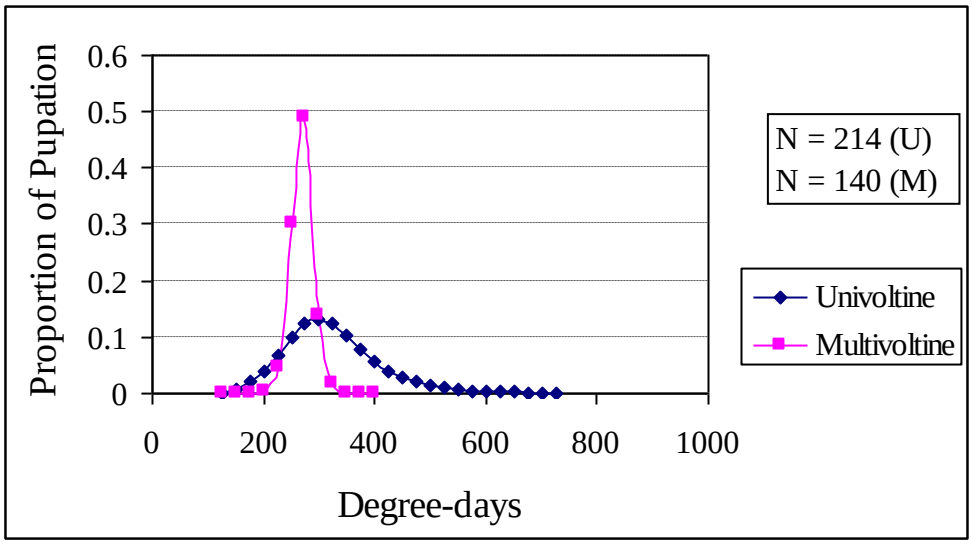


Figure 2.5: Prediction curve for degree-day requirements of non-diapausing 2002 F3 generation of univoltine and multivoltine *O. nubilalis* populations development from egg hatch to pupation at 25^0C and 18:6 (L:D) photoperiod.

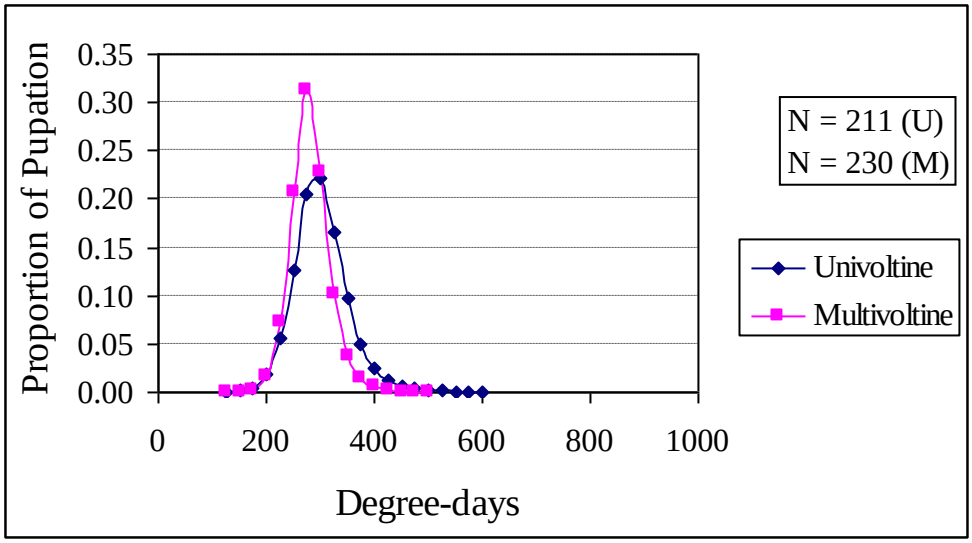


Figure 2.6: Prediction curve for degree-day requirements of non-diapausing 2003 F1 generation of univoltine and multivoltine *O. nubilalis* populations development from egg hatch to pupation at 25^0C and 18:6 (L:D) photoperiod.

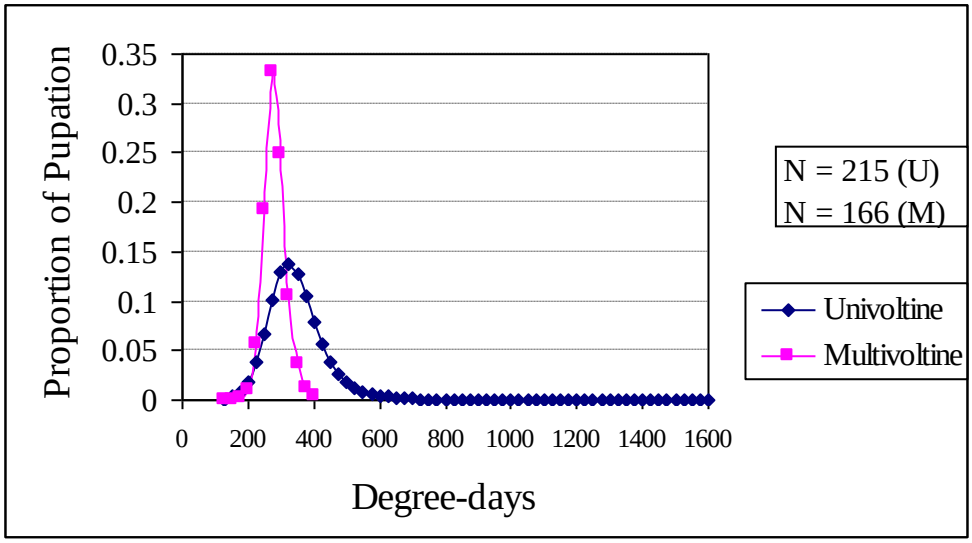


Figure 2.7: Prediction curve for degree-day requirements of non-diapausing 2003 F2 generation of univoltine and multivoltine *O. nubilalis* populations development from egg hatch to pupation at 25^0C and 18:6 (L:D) photoperiod.

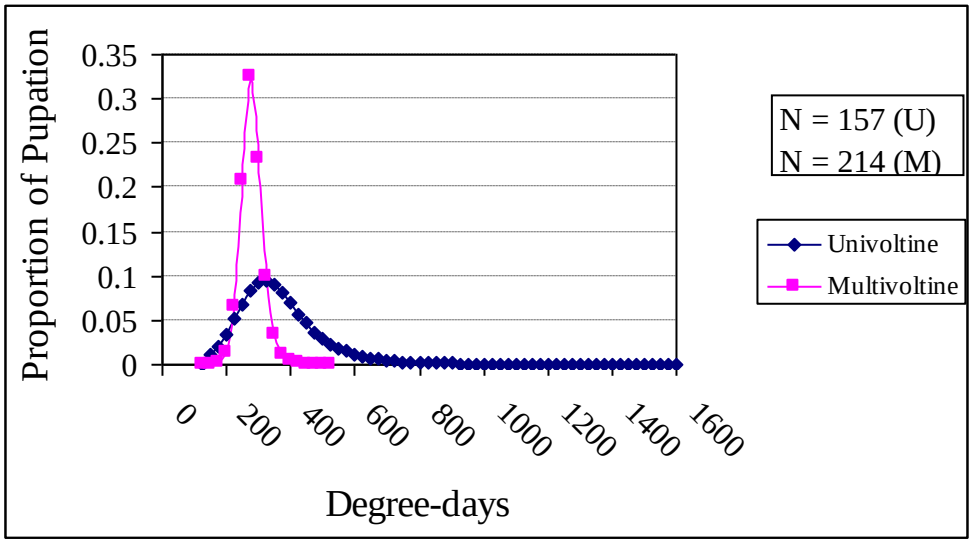


Figure 2.8: Prediction curve for degree-day requirements of non-diapausing 2003 F3 generation of univoltine and multivoltine *O. nubilalis* populations development from egg hatch to pupation at 25^0C and 18:6 (L:D) photoperiod.

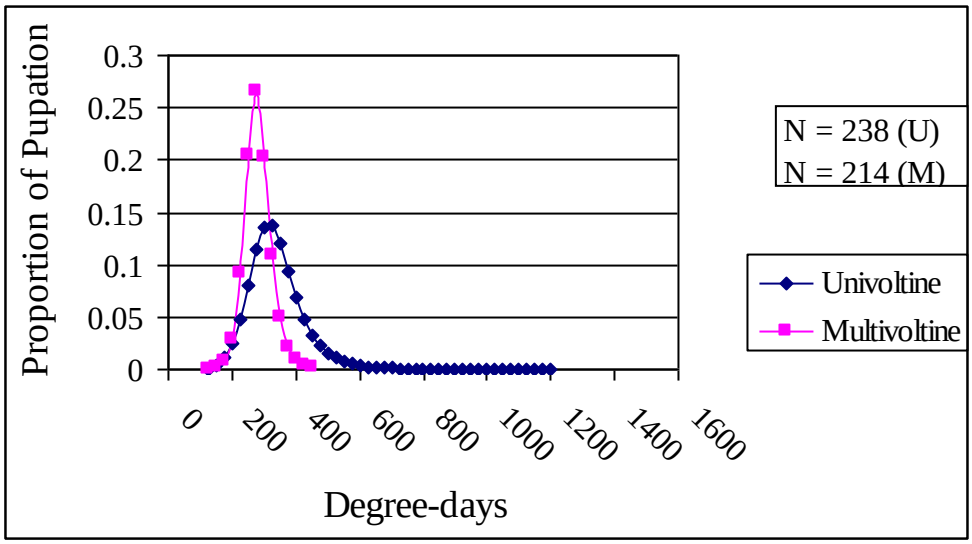
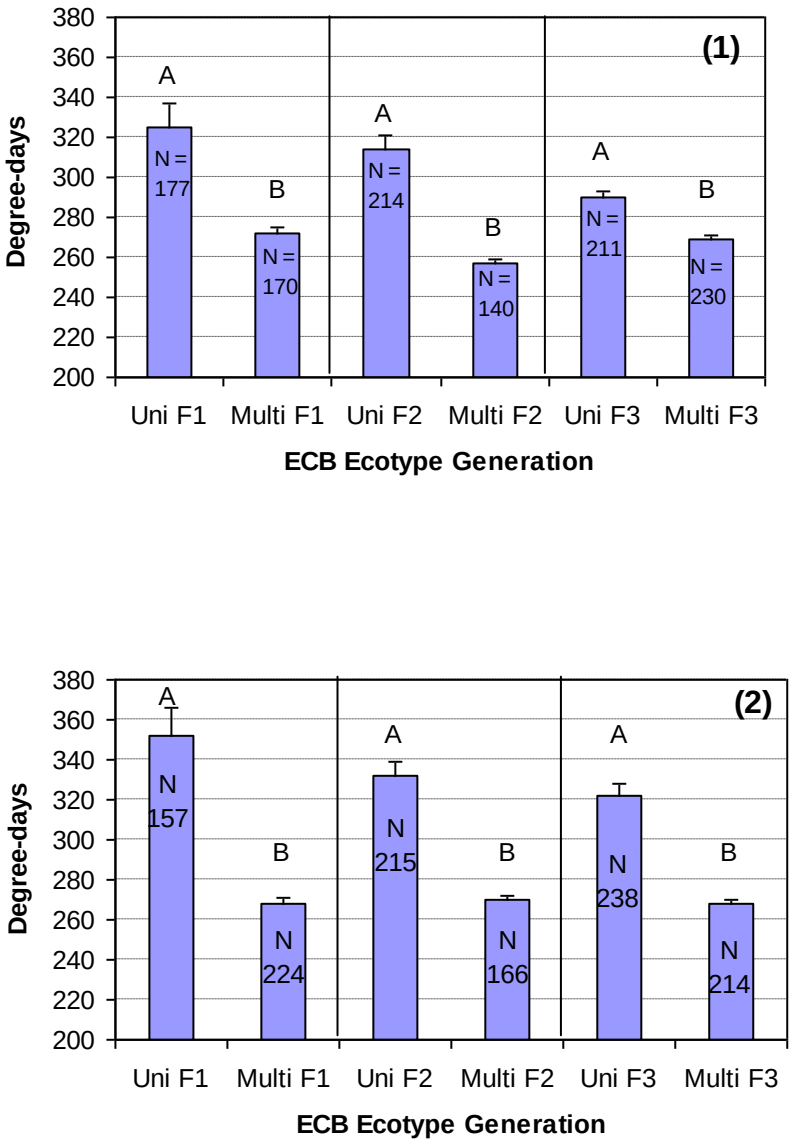


Figure 2.9: Comparison of mean degree-day requirements of non-diapausing F_1, F_2, and F_3 generations of univoltine and multivoltine *O. nubilalis* ecotypes reared in laboratory established from the (1) 2002 and (2) 2003 parent populations of central PA.



* Means with the same letter are not significantly different

Table 2.1: Estimated and proportion of multivoltine and univoltine types at the Rock spring site in Central Pennsylvania, USA along with cause of mortality.

Years	Locations in PA	Number of ECB collected	# of ECB pupated	# of ECB parasitize	# of natural death	Pupation started (DDA)	Pupation ended (DDA)	% of ECB pupated in ≤350 DD*	% of ECB pupated in >350 DD	50% population pupated at (DD)	95% population pupated at (DD)
2002	Rock spring	127	77	27	23	79.90	696.80	58.00	42.00	300.0	604.9
2003	Rock spring	448	352	54	42	48.56	1360.7	60.22	39.78	296.0	597.0
2004	Rock spring	359	253	48	58	71.06	1200.0	41.00	59.00	403.0	708.5
2005	Rock spring	422	242	82	98	75.00	1239.0	38.00	62.00	425.0	648.0
2005	Landisville	117	66	42	9	18.33	405.0	95.0	5.0	218.3	350.0
2005	Erie	75	54	9	11	75.0	775.0	33.0	67.0	400.0	625.0
2005	Bardford	85	68	4	13	75.0	750.0	76.0	24.0	237.5	462.5

* 350 seasonal degree-days was the estimated break between multivoltine and univoltine population.

Table 2.2: Non-diapause degree-days requirements and proportion of pupation of *O. nubilalis* larvae collected from the 2002 parent population. Larvae reared in artificial diet in the environmental chamber maintaining at 25^0C and 18:6 L:D photoperiod.

Voltinism and Generation	N	Pupation Started	Pupation Ended	Mean (±SE)	50% Pupation Completed at	95% Pupation Completed at
Univoltine F1	177	237.5	962.5	325.5 (11.2)	287.5	512.5
Multivoltine F1	170	212.5	362.5	272.2 (2.86)	262.5	337.5
Univoltine F2	214	212.5	700.0	313.8 (7.15)	287.5	637.5
Multivoltine F2	140	218.0	378.0	257.3 (1.76)	258.0	288.0
Univoltine F3	211	212.5	562.5	289.5 (3.8)	287.5	412.5
Multivoltine F3	230	212.5	462.5	268.7 (2.51)	262.5	325.0

Table 2.3: Non-diapause degree-days requirements and proportion of pupation of *O. nubilalis* larvae collected from the 2002 parent population. Larvae reared in artificial diet in the environmental chamber maintaining at 25^0C and 18:6 L:D photoperiod.

Voltinism and Generation	N	Pupation Started	Pupation Ended	Mean (±SE)	50% Pupation Completed at	95% Pupation Completed at
Univoltine F1	157	250.0	1612.5	352.4 (13.9)	312.5	487.5
Multivoltine F1	224	212.5	462.5	268.1 (2.44)	262.5	337.5
Univoltine F2	215	237.5	1050.0	332.3 (6.32)	312.5	512.5
Multivoltine F2	166	212.5	387.5	269.8 (2.35)	275.0	325.5
Univoltine F3	238	250.0	987.5	322.1 (6.07)	300.0	450.0
Multivoltine F3	214	225.0	675	267.64 (2.76)	262.5	325.5

Chapter 3:

Effects of post-diapause developmental degree-dayS on reproductive parameters of temporally separated and spatially isolated univoltine and multivoltine *Ostrinia nubilalis* populations in Pennsylvania, USA.

3.1 Introduction:

The European corn borer, *Ostrinia nubilalis* (Hubner) is one of the most important pests of corn (*Zea mays* L) in the North America (Mason et al. 1996). The species was native to Europe, was accidentally introduced in the U.S. during 1909-1914 (Vinal 1917). After few years of introduction, during 1919 the insect started expressing voltinism diversity within the *O. nubilalis* species (Felt 1921). Pheromone analysis of the filed collected female moths determined the existence of three sex pheromone and voltine populations of *O. nubilalis* in North America: (1) bivoltine Z (2) bivoltine E, and (3) univoltine Z populations (Roelofs et al. 1985, Hudon et al. 1989). In most parts of North America only one voltine type is dominant whereas there are a few regions where populations having a heterogenous response to the E and Z pheromonal biotypes were found to be sympatric. Based on the number of generations produced in a single year, Showers et al. (1975) described three ecotype populations of *O. nubilalis*: (1) a univoltine or northeren type (2) a bivoltine or central type, and (3) a multivoltine or southern type. Early researchers observed variations in the degree day requirement for peak pupation (50% pupation) among the three voltine or ecotype populations (Showers 1979, 1981).

Emergence periods of over wintering populations depend upon the post-diapause degree day or heat unit accumulation in the spring season. In central Pennsylvania, both univoltine and multivoltine ecotypes co-exist and the two ecotypes emerge in distinct periods in a growing season (Calvin and Song 1994). First generation post diapause

multivoltine populations emerge early in the growing season (late May to mid June) and a second generation emerges in the late summer (mid August) while univoltine populations emerge later in the spring season (mid July) between the two multivoltine generations.

Ostrinia nubilalis lifecycle consists of four life stages: egg, larva, pupa, and adult. Among the four life stages, the larval stage is the longest and most of the growth and development happens in this stage. During larval development *O. nubilalis* completes five instars. In the fifth instar all the healthy larvae either continue their development toward to pupation or enter into the state of arrested development called diapause. Diapause in *O. nubilalis* is facultative, believed to be controlled by an environmental cue such as photoperiod, temperature, and perhaps by the nutritional quality of the food rather then control entirely by the genetic characteristics of the organism. During spring season when the atmospheric temperature reaches above the base developmental threshold temperature (12.5^{o}C) diapausing *O. nubilalis* larvae resume their development.

Variation in voltinism or ecotype emergence period may have consequences on their offspring's development and growth pattern. Host plant selection in *O. nubilalis* is decided by the female moth not by the larvae, but the larva can select the plant part on which to feed. Upon emergence first generation multivoltine females prefer to oviposite in corn fields planted earlier in the season with the plants being about 46cm tall (Spangler and Calvin 2001). First generation multivoltine larvae primarily feed on the tissues of young corn plant, while larvae from the 2^{nd} generation feed on mature reproductive stage corn plants. On the other hand univoltine larvae feed on the succulent vegetative tissue of growing corn plants.

The selection of a feeding site is influenced by the chemical and physiological composition of the host plant as well as the behavioral responses of the insect to the plant's environment (Neiswander 1928). As the developmental stages of the corn plant change, the feeding preferences of the infesting corn borers also change (Beck 1960). Beard (1941) suggested that not all the tissues in a plant are equally preferable to the larvae and found differential feeding behavior. Earlier in their life stages, larvae of the 1st generation *O. nubilali*s are leaf feeders, becoming stalk borers in the fifth instar (Beck 1956a, Guthrie 1971), while 2nd generation larvae hatch on plants that have tasseled and silked and feed initially on pollen, silk and young ear husk eventually entering the ear itself (Beck 1956a, Guthrie 1969, 1970, Guthrie et al. 1980). However, neonates of univoltine ecotypes feed primarily on the leaf sheath and collar.

Chemical composition of the corn plant varies between the stages of plants. DIMBOA (2,4-dihydroxy7-methoxy-1,4-benzoxazine-3-one), a chemical compound found in the early stage corn leaves is believed to have an anti-feedent effect against first generation *O. nubilalis* (Klun and Brindley 1966, Russel et al. 1975), whereas it is not a major factor for the 2nd generation *O. nubilalis* population as levels of DIMBOA concentrations decrease in mature plants. Beck (1956a) found *O. nubilalis* feeding to be stimulated by the presence of sugar in the accessible tissues such as whorl, tassel, and leaf sheath. Other chemicals such as phenolic acid derivatives (flavones and flavonols) are found in corn tissues at varying levels and are believed to have antagonistic effects on *O. nubilalis* feeding (Ceska and Styles 1984, Abouzaid et al. 1993, Bergvinson et al. 1994). A number of amino acids were also found to influence (stimulant or deterrent) the feeding of European corn borer larvae (Beck 1956b, Beck and Henec 1958, Beck 1960).

The variation in emergence time between univoltine and multivoltine ecotypes and the potential consequences of the biochemical and behavioral feeding characteristics might influence *O. nubilalis* fecundity and other adult reproductive parameters. In France, sympatric *O. nubilalis* populations feeding on different host plants have different larval weights (Calcagno et al. 2007), and these populations on different hosts are morphologically indistinguishable (Bourguet et al. 2000, Martel et al. 2003, Leniaud et al. 2006). Although unknown for *O. nubilalis*, female codling moth from different host races demonstrated varied pupal weight and those pupal weights were positively correlated with higher levels of adult fecundity (Cisneros and Barnes 1974). A laboratory investigation by Myers et al. (2006) found higher pupal weight and a longer oviposition period in Oriental fruit moth, *Grapholita molesta*, reared in apple than reared in peach. While it is unknown what influences the growth and development of ecotype populations of *O .nubilalis*, it is possible there could be some fitness advantage of having higher larval and pupal weight.

This study was designed to address the following questions: (1) Do the larval and pupal weights of the ecotype populations differ? (2) Do the variable female pupal weights have an effect on fecundity? (3) Is adult female longevity (pre-oviposition, oviposition, and post-oviposition period) affected by the larval and pupal weight? To investigate those questions, in 2004-05 post-diapause larvae were collected from a temporally separated sympatric ecotype region in Centre county (Rock spring), PA. In 2005 three additional locations of spatially separated ecotype populations across Pennsylvania were added to this study. Non-diapause pupal weights were also studied with the first generation (F1)

univoltine and multivoltine populations established from the field collected over-wintered parent population.

3.2 Materials and Methods:

3.2.1 ECB larval and pupal weight studies:

Temporally separated post-diapause ecotype populations: Over wintered European corn borer larvae collected in 2004 and 2005 at Rock Spring, PA were used in the experiment. Larval weight was measured in the laboratory within a couple of hours of collection by using an electronic analytical balance (Sartorious, Germany). After weighing, a larva was placed individually into a 30ml plastic cup without food. A small water soaked cotton ball was added to provide moisture control. Once a larva pupated, its weight was measured between 24-36 hrs. A new pupa was allowed to age for 24-36 hrs after pupation to avoid injuring it while it was soft and delicate. The pupa was then transferred to a new cup for adult emergence.

Univoltine and multivoltine populations were segregated by following the methods described by Calvin and Song (1994). By using a 150 degree-day buffer larvae pupating ≤300 degree-day accumulations (from 1st January) were considered multivoltine. Larvae pupating at ≥ 451 degree-day accumulation were considered univoltine. Univoltine and multivoltine colonies were established in the laboratory by following the same procedure as described in the colony establishment section in chapter 2. Both univoltine and multivoltine adults were placed separately into mating cases for oviposition. Egg masses were collected and reared as described earlier chapter. Sympatric univoltine and multivoltine larval and pupal weight data were reported in table 3.1

Spatially isolated post-diapause ecotype populations: A multi-location spatially separated (allopatric) univoltine or multivoltine post-diapause larval and pupal weight study was done to validate the sympatric population results in central Pennsylvania. In 2005, post-diapause larva were collected from three locations: (1) Landisville, PA a location predominantly a multivoltine region (2) Erie, PA predominantly a univoltine region, and (3) Bradford, PA a univoltine region.

Post-diapause larval weight was measured after field collection from fields and then put in the laboratory growth chamber for pupation maintaining the same environmental condition as the sympatric population experienced. Pupal weight was measured and was allowed for adult eclosion. Allopatric univoltine and multivoltine larval and pupal weight data were reported in table 3.2.

Non-diapause (F1) ecotype populations: Around two hundred and ten, first generation (F_1) 24-h old larvae from each of the multivoltine and univoltine populations were selected randomly from the 2005 field collected population. The selected larvae were then transferred into individual diet cups containing approximately 6 mg of diet in each cup. The diet was prepared by following the same instructions as described in the previous chapter. Cups were placed in the environmental growth chamber (Revco model no. RI-23-555-A). The chamber was maintained at the same temperature, humidity and photoperiod as the larvae's parents experienced (25^0C, 18:6 L:D, and 60% humidity). Date of pupation and weight of the individual pupa were recorded by following the same procedure described above. The pupae were allowed to molt into adults. Adult sex and date of emergence were recorded. Individual males and female larval and pupal weight

data of the univoltine and multivoltine populations were determined after adult eclosion. During the whole experiment the growth chambers temperature was measured continually by placing a calibrated thermometer to assure the accuracy of the growth chambers temperature reading.

For all the larval and pupal weight data, plots of cumulative univoltine and multivoltine pupation frequency over time (accumulated degree-days) were constructed (apenendix 2. figure 3.10-3.18). Differences in multivoltine and univoltine post-diapause and non-diapause larval and pupal weights were analyzed by using two sample T-tests (alpha <0.05) (Minitab 2000) (table 3.3 and 3.4). Significant differences were observed between male and female European corn borers larval and pupal weight. Beck (1989), found that female *O. nubilalis* pupae were normally larger than male pupae. To avoid larger standard error (SE) in all larval and pupal weight studies we analyzed the male and female data separately. Statistical analyses for larval and pupal weight were reported in figure 3.1-3.9.

3.2.2 Post-diapause reproductive parameter studies:

Temporally isolated post-diapause ecotype populations: During the second week of April to the first week of May 2003, three hundred and fifty six diapausing 5^{th}-instars larvae were collected from the cornfield in Rock Spring, Pennsylvania. Collected larva were transferred into environmental growth chamber in a diapause breaking environmental condition (25^0C, 18:6 L:D, and 60% humidity). The rearing procedure was the same as described in the colony collection and rearing section in chapter 2. The collected population was divided into eleven groups based on the degree-day requirement

for pupation of each larva. Groups were defined by 50 degree-days intervals starting from 50 and ending at 600 degree-days. Each group was assigned by a letter from A to K. Although, some of the field-collected larvae pupated above six hundred degree-days, (i.e up to 1360 degree-day), feasible mating pairs were obtained only or the intervals between 50 – 600 degree-days. After six hundred degree-days, numbers of emerging adults were so low and irregular that establishing a viable mating partner was not possible. From each group at least four pairs of males and females were transferred into four different mating chambers.

The *O. nubilalis* adult mating chamber was a cylindrical cage 3.5 inches in height and 3.5 inches in diameter. The cylinder was made of 256-mesh size aluminum screen with the upper side open. Mated pairs were established with the closest proximity (± 10 degree-day) of their degree-days requirement - if one parent emerged after a specific degree-days the partner was selected from adults that emerged within the same degree-day interval. A total of 51-mated pairs were transferred into 51 cylindrical chambers. A small cotton ball soaked with 15% honey solution was placed inside the mating chamber. The upper ends of the chambers were closed with a 64-mesh size aluminum screen lid. Wax paper for oviposition was placed on top of the screen lid. The cylindrical chambers were then placed into a walk in growth chamber at 25^0C, 18:6 L:D, and more then 95% humidity.

The number of egg masses laid on a particular date were marked, counted, and recorded each morning. The wax paper containing the egg masses was removed after 2 to 3 days depending upon the intensity of the oviposition. Removed egg masses were kept in a Mason jar with a moist towel in the bottom. Within two to three days the egg masses

reached the black head stage, a sign of a viable egg mass about to hatch. Then the number of black-headed larvae per egg masses was counted under the microscope and recorded. The number of days before oviposition (pre-oviposition), the number of ovipositing days (egg laying period), and the number of days after oviposition (post-oviposition) was recorded for each of the females. Male and female longevity was also recorded. The pre-oviposition period was calculated by subtracting the date of first oviposition from the date of female emergence. Oviposition period was calculated by subtracting the date of first oviposition from the date of last oviposition for individual females. The post-oviposition period was calculated from the difference between the last oviposition date and the date the females deceased. Female longevity was calculated as the days between adult emergence and death. Fecundity was calculated by counting the egg masses and the number of eggs per egg mass laid by a female over the oviposition period. Reproductive parameter differences between univoltine and multivoltine were analyzed by using SAS procedure (SAS Institute 1990). A normality test was conducted along with each of the parameters tested to evaluate whether the data was normally distributed. If the data were normally distributed a General Linear Model (GLM) procedure was conducted to calculate the F-value and the probability of rejection. If the normality hypothesis was rejected then the data were transformed into log values to normalize the data for the GLM procedure. If the normality hypothesis was rejected for the log-transformed value, then a nonparametric (GENMOD) procedure following the Poisson distribution was used to compare the tested parameter among the eleven groups. If significant variation was found among groups, a pair wise comparison was conducted to identify which group or groups were significantly different. A nonparametric one-way procedure (Wilcoxon two-sample

test) was conducted to compare reproductive parameters between univoltine and multivoltine populations. Reproductive parameter comparison between degree-day groups and sympatric univoltine and multivoltine populations were reported in table 3.5 and 3.6.

3.3 Results:

3.3.1 ECB larval and pupal weight studies:

Temporally separated post-diapause ecotype populations: Larval weight comparisons between the univoltine and multivoltine population in 2005 showed significantly higher larval weights in univoltine males than larval weights of multivoltine males collected at Rock Spring, PA ($T = -2.04$, $p = 0.044$, $df = 106$) (figure 3.2). Similarly, post-diapause univoltine females larval weights were significantly higher than the multivoltine females larval weights ($T = -3.22$, $p = 0.002$, $df = 69$) (figure 3.3). Larval weights of univoltine males were an average 6.2% heavier than the multivoltine ecotype males. On the other hand, female larval weights of the univoltine ecotype were an average 11.6% heavier than the female larvae of the multivoltine ecotype (table 3.1).

Univoltine female pupal weight was significantly higher than multivoltine female pupal weight for the 2005 field collected post-diapause population ($T= -2.25$, $p = 0.013$, $df = 67$) (figure 3.3). However, mean pupal weights of univoltine and multivoltine males were not significantly different ($T= -1.53$, $p = 0.125$, $df = 110$) (figure 3.3). Although, male pupal weights of the univoltine and multivoltine populations were not statistically different, univoltine male pupae were on average 5.2% heavier than multivoltine males.

On the other hand, univoltine female pupae were an average 9.5% heavier than the multivoltine female pupae (table 3.1).

During the pupation period, the univoltine male population reduced its larval weight an average 13.6% to become a pupa in compared to 12.7% for multivoltine males. On the other hand, univoltine females reduced the larval mass by 12.8% compare to 11.2% for the multivoltine females. It was observed that irrespective of males and females, univoltine larva lose slightly more mass (0.83 – 1.67%) than multivoltine larva during the pupation process.

In 2004, only pupal weights of the univoltine and multivoltine post-diapause populations were compared. Male pupae of the univoltine ecotype were significantly heavier than the male pupae of the multivoltine ecotype ($T = -2.01$, $p = 0.047$, $df = 91$). Similarly, the mean female pupal weight of univoltine ecotype was significantly higher than pupal weight of the multivoltine ecotype ($T = -2.13$, $p = 0.039$, $df = 45$) (figure 3.1). In 2004, univoltine male pupae were an average 9.0% heaver than the multivoltine male pupae while the univoltine female pupae were an average 13.4% heavier than the multivoltine female pupae.

Spatially isolated post-diapause voltine populations: Mean larval weight of the Landisville population, a region dominanted by multivoltine European corn borer, was significantly lower than the Erie population, a region of predominant by the univoltine ecotype. The mean males and females larval weights of the univoltine Erie population was an average 20.7% and 15.6% heavier than those of the multivoltine Landisville population, respectively ($T = -4.96$, $p = 0.0001$, $df = 43$ and $T = -2.82$, $p = 0.007$, $df =$

45) (figure 3.4). Erie male pupal weights were significantly higher (12.1%) than the Landisville male pupal weights (T = -2.82, p = 0.007, df = 44). Similarly, Erie females pupal weights were significantly higher (8.6%) than the Landisville females pupal weights (T = -2.02, p = 0.049, df = 47) (figure 3.4 and table 3.2).

Another comparison of the multivoltine Landisville population with a predominant univoltine population collected from Bradford county region resulted a significantly higher (14.3%) male larval weights for univoltine populations (T = -3.58, p = 0.001, df = 54). Female larval weight of *O. nubilalis* from Bradford county was also significantly higher (15.6%) than that of the Landisville population (T = -3.29, p = 0.002, df = 41). The male pupal weight of theBradford county population was significantly higher than that of the Landisville population (T = -2.26, P = 0.028, df = 55). Similarly, females pupal weights of the Bradford population were 10.6% higher than that of Landisville population (T = -2.49, p = 0.016, df = 52) (figure 3.5, table 3.2).

Comparison between the mean male and female larval weight of the two univoltine regions (i.e. Erie and Bradford counties) suggested no significant difference (T = 1.32, p = 0.191, df = 52 and T = -0.26, p = 0.798, df =57, respectively, for males and females). The pupal weights of these two populations were also not significantly different (T = 0.53, p = 0.597, df = 52 and T = -0.48, p = 0.632, df = 63, respectively, for males and females). Larval and pupal weights of those two spatially separated univoltine populations were almost similar but they were significantly different from the spatially separated multivoltine population (figure 3.6, table 3.2).

Non-diapause (F1) ecotype populations: A pupal weight comparison analysis between non-diapause first generation (F1) univoltine and multivoltine 2005 ecotype populations reared on artificial diet in a laboratory growth chamber suggested similar pupal weight trends between these two ecotypes. No significant difference was observed between non-diapause univoltine and multivoltine males pupal weight ($T = 0.68$, $p = 0.495$, $df = 150$). Non-diapause univoltine pupae were only 1.2% heavier than the non-diapause multivoltine pupae. Non-diapause univoltine female pupal weight was also not significantly different from the non-diapause multivoltine female pupal weight ($T = -1.69$, $p = 0.092$, $df = 159$). Non-diapause univoltine female pupae were an average only 3.0% heavier than the non-diapause multivoltine female pupa (figure 3.7, table 3.3).

Although no significant weight differences were found between non-diapause univoltine and multivoltine pupa, significant differences were observed between the non-diapause (F_1 lab population) and post diapause (parent) field populations. A comparison analysis was conducted between the non-diapause and post-diapause pupal weight data for the 2005 *O. nubilalis* population. The mean pupal weight of the non-diapause univoltine male population was significantly higher (16.2%) than that of the post-diapause univoltine males ($T = 5.68$, $p = 0.0001$, $df = 119$). Similarly, non-diapause univoltine females pupal weight were significantly higher (14.3%) than the post-diapause univoltine females pupal weight ($T = 6.10$, $p = 0.0001$, $df = 165$) (figure 3.8, table 3.4). Non-diapause multivoltine males pupal weight were also significantly higher (20.79%) than the post-diapause multivoltine males pupal weights ($T = 7.78$, $p = 0.0001$, $df = 75$). Similarly, Non-diapause multivoltine females pupal weights were significantly higher

(29.0%) than the post-diapause multivoltine females pupal weights (T = 8.17, p = 0.0001, df = 54) (figure 3.9, table 3.4).

3.3.2 Post-diapause reproductive parameter studies:

Table 5 and 6 summarize the results of the eight reproductive parameters tested for 51 families of European corn borer categorized under eleven degree-day groups. The pre-oviposition period among the 51 females varied between one to nine days, but most of the females initiated oviposition within two to three days of their emergence. Among the 51 females tested, only one female initiated oviposition as early as one day and eight females initiate oviposition three days after adult emergence. Among the eleven European corn borer groups established based on their post diapause degree-day requirements before pupation, the mean pre-oviposition period varied between 2.0 - 4.3 days. However, variation in pre-oviposition period was not statistically different among the eleven European corn borer groups (F = 0.89, DF = 10, P = 0.552) (table 3.5). When the first six group (A-F) which comprise the multivoltine ecotype were compared with the last five group (G-K) which comprise the univoltine ecotype, there were no significant difference in pre-oviposition period between multivoltine and univoltine ecotypes (Wilcoxon statistics = 612.5, P = 0.777) (table 3.6). Similarly, mean oviposition period length was not significantly different among the groups (F = 0.22, DF = 10, P = 0.992) and no significant difference was found between univoltine and multivoltine ecotypes (Wilcoxon statistics = 565, P = 0.538).

The post-oviposition period length varied between 0 to 7 days among the *O. nubilalis* females. A significant difference was observed among the 11 post-diapause

interval groups (Chi-square = 20.27, DF = 10, P = 0.026). A pair wise comparison found that group G (mean = 0.50 days) had a significantly shorter post-oviposition period from the other groups. Although a significant difference in post-diapause period was observed among the groups, no significant difference was found between the univoltine and multivoltine ecotype groups (Wilcoxon statistics = 16.48, P = 0.164).

European corn borer female longevity varied between 5 and 19 days, but female longevity were not significantly different among the groups (F = 0.71, DF = 10, P = 0.707). No significant difference was observed between univoltine and multivoltine populations (Wilcoxon = 555.50, P = 0.427).

The number of egg masses per female varied from 6 to 55 among the females tested, but no significant difference was observed among the 11 groups (F = 0.29, DF = 10, P = 0.98). When groups were categorized into univoltine and multivoltine, there was no significant different among the ecotype populations (Wilcoxon = 524, P = 0.17).

Mean number of egg masses per day was not significantly different among the groups (F = 0.91, DF = 10, P = 0.53). No significant variation was found between univoltine and multivoltine population (Wilcoxon statistics = 568.50, P = 0.58).

Mean number of eggs per egg mass varied between females (6.68 to 28.71 eggs/egg mass) but these variations were not statistically significant among the *O. nubilalis* groups (F = 1.36, DF = 10, P = 0.233). No significant different in number of eggs per egg mass was observed between univoltine and multivoltine ecotypes (Wilcoxon statistics = 647.50, P = 0.35). Although, the total number of eggs laid by individual females had huge numerical variation (97 to 854 eggs/female), the mean number of eggs laid by a particular degree-day group was not significantly different between groups (F =

0.48, DF = 10, P = 0.891). There were no significant variation in number of eggs laid between univoltine and multivoltine ecotypes (Wilcoxon statistics = 585, P = 0.813).

3.4 Discussions:

The present study indicated significant differences in post-diapause larval and pupal weight between univoltine and multivoltine ecotype population. In 2004 and 2005, we observed similar trends in female pupal weight in the field collected post-diapause population. In both the years univoltine female pupae were significantly heavier in weigh than the multivoltine pupae. In 2004, univoltine male pupae were significantly heavier (9.0%) than the multivoltine pupae. Although, no statistical difference were observed between 2005 post-diapause univoltine and multivoltine males pupal weight, univoltine male pupa were 5.2% heavier than the multivoltine male pupa (table 3.1). From the above results, it was observed that there were consistently higher larval and pupal weight in univoltine than multivoltine post diapause populations and these differences were consistent in the male and female populations of the univoltine and multivoltine ecotypes.

It was also evident that in spite of the long-term diapause conditions, univoltine individuals do not compromise their larval and pupal weight. It was observed that regardless of sex (males nd females) univoltine larva lose slightly more weight (0.83 – 1.7%) than multivoltine individuals during the pupation period, despite a longer time spent overwintering. This finding was in contrast to the assumption that an individual that is dormant for an extended period would lose more weight than an individual that had a shorter time from reaching the mature larval stage and spring pupation. It is possible that univoltine individuals may gain more larval body mass before going to diapause in their

5^{th} instars and also may have a mechanism to minimize body mass lose during the long term pre-pupation period.

Higher weight gain in univoltine larva may be the results of food quality difference available during the larval feeding period. It is known from previous studies that univoltine and multivoltine adults emerge at different time during the growing season, thus feed on different host plant stages.

Yield loss due to crop damage depends on synchrony between the presence of insect life stage and plant phenology. In the U.S. major corn growing regions, European corn borer has two generations per year with the first generation feeding primarily on leaf tissue and the second generation feeding primarily on leaf sheath, shank, and stalk tissue (Mason et al. 1996). First generation multivoltine European corn borer prefer earlier planted cornfields that are about 46 cm tall as oviposition sites and second generation female moth prefers more succulent late planted or larger corn plant as their oviposition site and lay eggs around the ear leaf (Spangler and Calvin 2001).

Study with the spatially isolated ecotype population also confirms the higher larval and pupal weight in the univoltine ecotype population. Larval and pupal weight of Erie and Bradford individuals (univoltine dominated regions) were similar and significantly heavier than the individuals from the multivoltine region near Landisville, PA. Although, post-diapause studies suggested significant larval and pupal weight differences between univoltine and multivoltine ecotypes, when reared on artificial diet in controlled environment in the laboratory growth chamber non-diapause pupal weight studies showed no significant differences between the ecotypes. However, it was observed that irrespective of ecotypes and sex, non-diapause pupal weight were 14.3 to

29.0% heavier than the post-diapause pupal weight (figure 3.4-3.5, table 3.8). Similar pupal weight trend in both univoltine and multivoltine non-diapause populations reared in control condition suggested that weight gain during the larval development may not be a heritable trait but rather an environmentally influenced trait.

The difference between post-diapause and non-diapause pupal weight may suggest a diapause induced feeding pattern or growth in the fifth instar pre-diapause ECB larvae under field conditions. Rearing both ecotype populations on a similar diet and environmental condition may rule out a possible genetic basis of weight difference and strengthen the possibilities of an environmentally induced and host plant related influence. These observations suggested that retaining of pupal weight might depend upon larval weight gain during the larval feeding period. Pupal weight differences between the non-diapause and post-diapause population might be the result of diet type and the nutritional quality of the diet. Significant larval weight differences between the univoltine and multivoltine regions might be the outcome of feeding on different stages of the corn plant, the corn tissues *O. nubilalis* larva feed on, and perhaps the environmental condition (temperature, photoperiod etc) of the region. Significant larval weight difference between the post-diapause temporally and spatially isolated univoltine and multivoltine population might have resulted from differential feeding regime during the corn-growing season. Studies on the reproductive parameters comparison between univoltine and multivoltine found no significant differences. No differences in the reproductive parameters may suggest the presence of random mating between the two overlapped ecotype. Future study with the distinct temporarily and spatially isolated *O. nubilalis* ecotype populations may reveal the assumption of speciation hypothesis.

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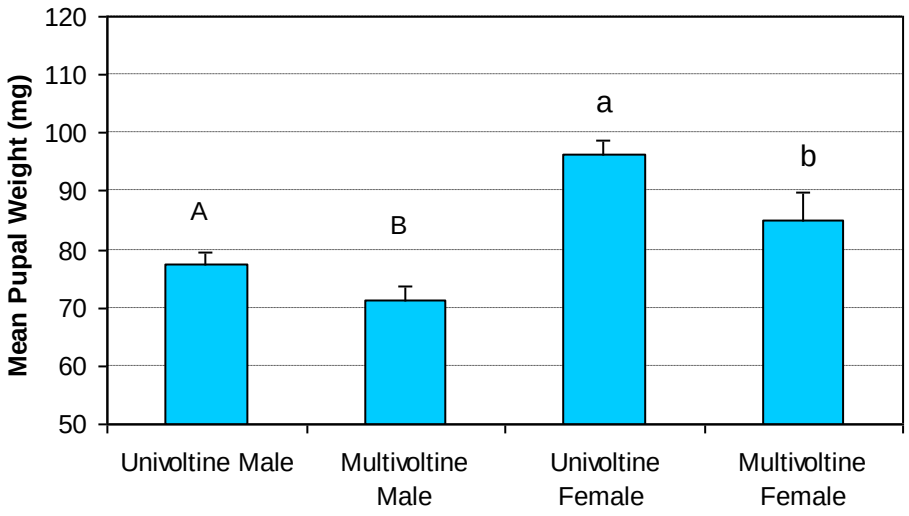
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Figure 3.1: Mean pupal weight of the 2004 post-diapause univoltine and multivoltine population measured after pupation in environmental chamber in laboratory in the Entomology department at Pennsylvania State University, PA.



* Means with the same letters are not significantly different

Figure 3.2: Mean larval weight of the 2005 post-diapause univoltine and multivoltine population measured after collected from fields of the Rock Spring, Pennsylvania.

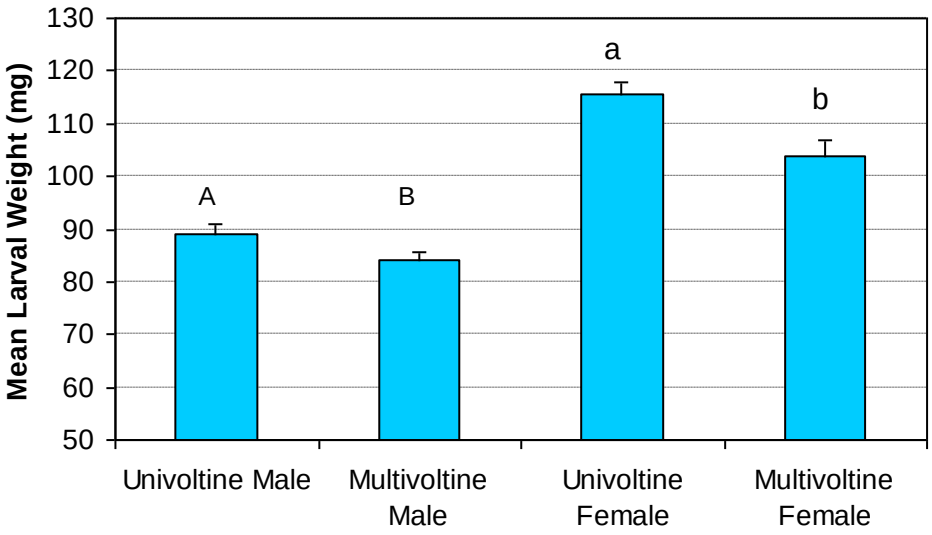
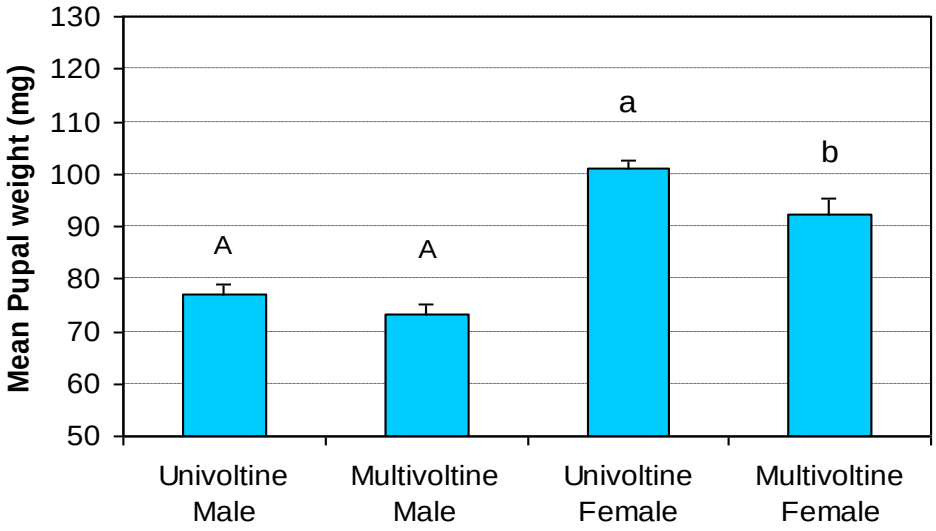


Figure 3.3: Mean pupal weight of the 2005 post-diapause univoltine and multivoltine population measured after pupation in environmental chamber in laboratory in the Entomology department at Pennsylvania State University, PA.



* Means with the same letters are not significantly different

Figure 3.4: Mean larval and pupal weight of the 2005 post-diapause *Ostrinia nubilalis* population collected from the regions of Erie and Landisville Pennsylvania, predominantly considered as the area of univoltine and multivoltine ecotypes respectively.

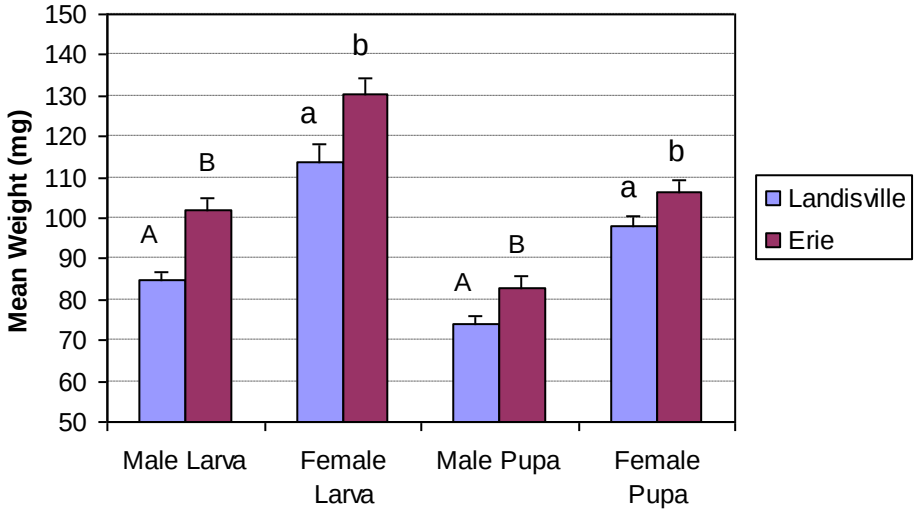
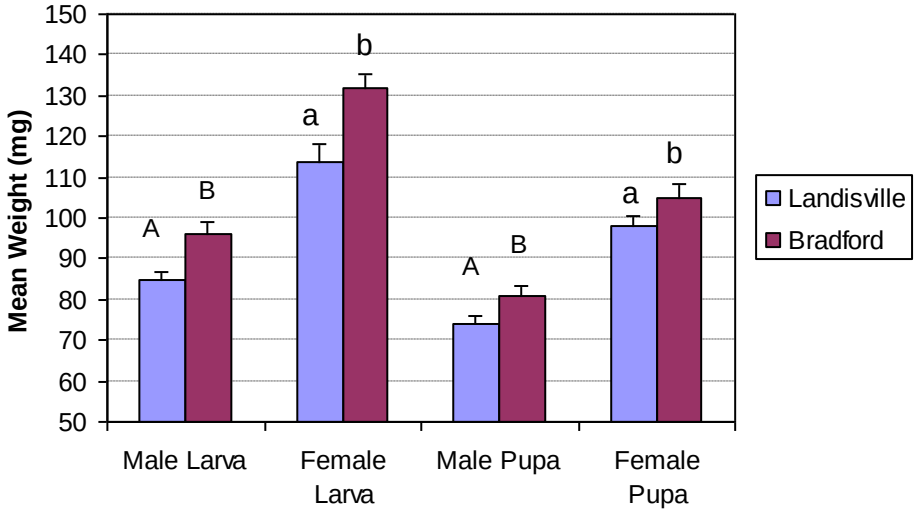


Figure 3.5: Mean larval and pupal weight of the 2005 post-diapause *Ostrinia nubilalis* population collected from the regions of Bradford and Landisville, Pennsylvania, predominantly considered as the area of univoltine and multivoltine ecotypes respectively.



***** Means with the same letters are not significantly different

Figure 3.6: Mean larval and pupal weight of the 2005 post-diapause *Ostrinia nubilalis* population collected from the regions of Erie and Bradford, Pennsylvania, predominantly considered as the area of univoltine ecotype.

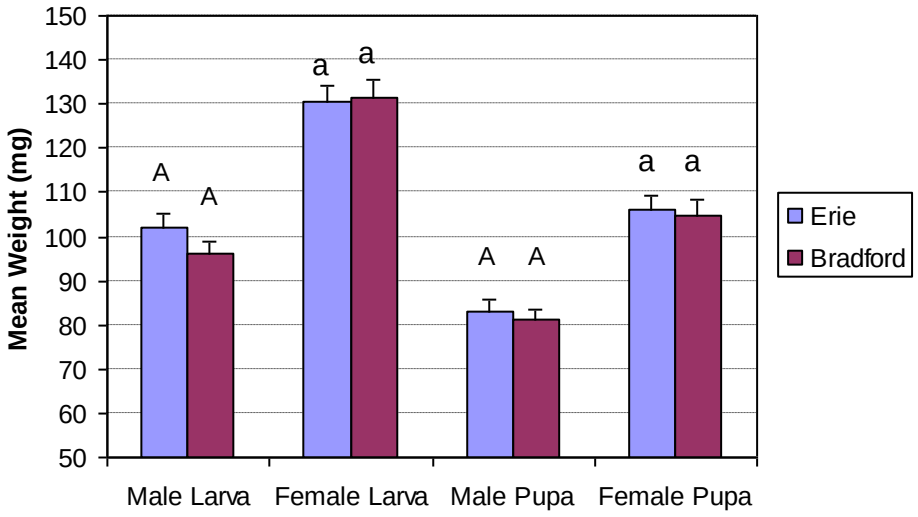
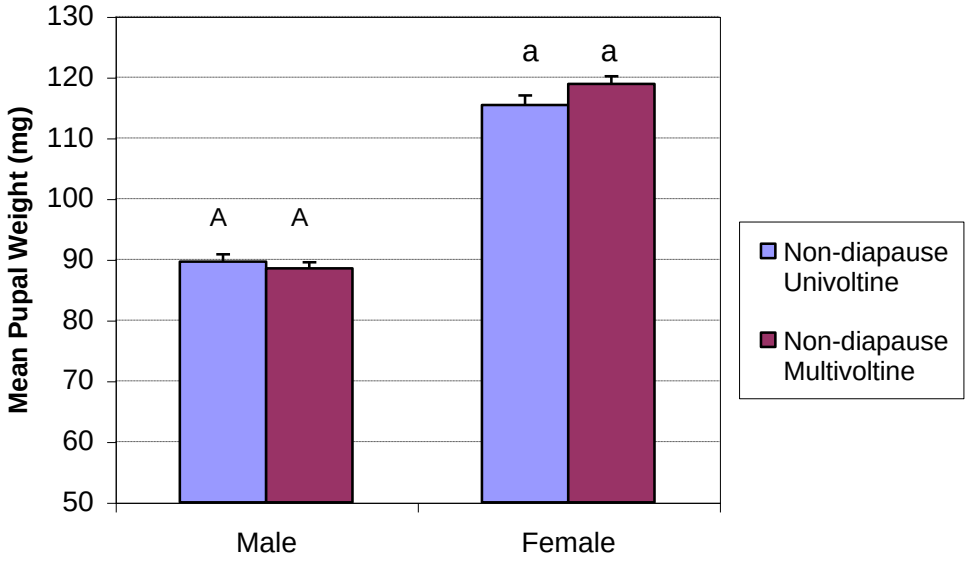


Figure 3.7: Mean pupal weight of the 2005 Non-diapause (F1) univoltine and multivoltine population measured after pupation in environmental chamber in laboratory in the Entomology department at Pennsylvania State University, PA.



* Means with the same letters are not significantly different

Figure 3.8: Mean pupal weight of the 2005 post-diapause and non-diapause (F1) univoltine population measured after pupation in environmental chamber in laboratory in the Entomology department at Pennsylvania State University, PA.

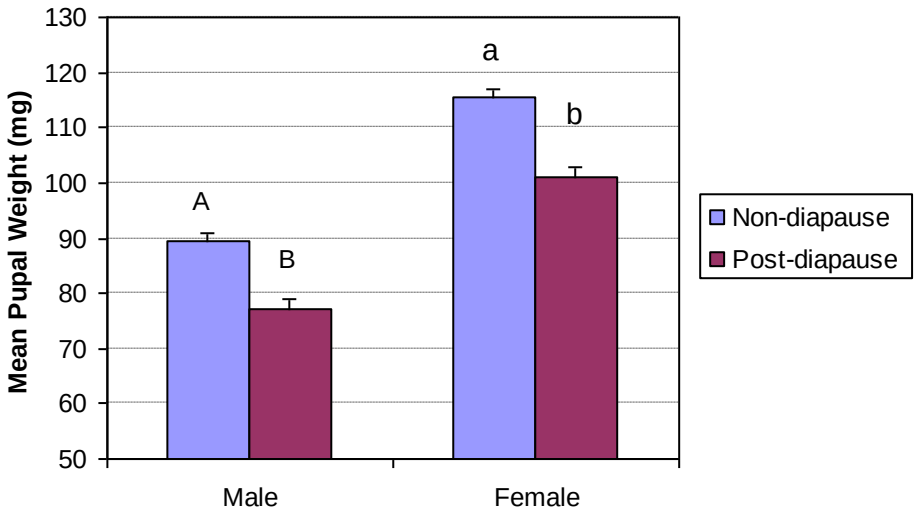
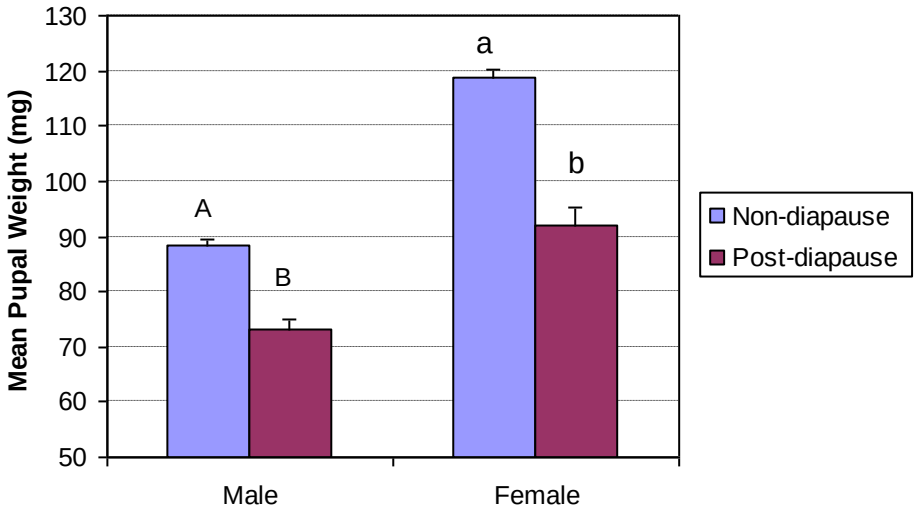


Figure 3.9: Mean pupal weight of the 2005 post-diapause and non-diapause (F1) multivoltine population measured after pupation in environmental chamber in laboratory in the Entomology department at Pennsylvania State University, PA.



* Means with the same letters are not significantly different

Table 3.1: Comparison of temporally separated univoltine and multivoltine larval and pupal weight of over-wintered *O. nubilalis* populations collected in central Pennsylvania.

Ecotype Compared	**Year**	**Life stage compared**	**Sex**	**N**	**Mean weight in mg (SE)**	**P-Value ***	**DF**	**Estimated difference (mg)**	**Weight Difference (%)**
Univoltine	2005	Larva	M	67	89.2 (1.9)	0.044	106	5.20	6.19
Multivoltine				47	84.0 (1.8)				
Univoltine	2005	Larva	F	82	115.8 (1.9)	0.002	69	12.01	11.56
Multivoltine				41	103.8 (3.2)				
Univoltine	2005	Pupae	M	67	77.1 (1.8)	0.125	110	3.85	5.18
Multivoltine				47	73.3 (1.7)				
Univoltine	2005	Pupae	F	81	101.0 (1.7)	0.013	67	8.85	9.54
Multivoltine				41	92.2 (3.0)				
Univoltine	2004	Pupae	M	62	77.5 (1.8)	0.039	91	6.35	9.00
Multivoltine				50	71.1 (2.6)				
Univoltine	2004	Pupae	F	57	96.3 (2.3)	0.012	45	11.41	13.42
Multivoltine				32	84.9 (4.8)				

* $P \geq 0.05$ are not significantly different

Table 3.2: Comparison of spatially isolated univoltine and multivoltine larval and pupal weight of over-wintered *O. nubilalis* population collected from Landisville, Erie, and Bradford region of Pennsylvania in 2005.

Ecotype Compared	**Life stage compared**	**Sex**	**N**	**Mean weight in mg (SE)**	**P-Value**	**DF**	**Estimated difference (mg)**	**Weight Difference (%)**
Landisville Erie	Larvae	M	38 25	84.7 (1.9) 102.2 (14.8)	0.0001	43	17.51	20.66
Landisville Erie	Larvae	F	22 29	113.8 (4.4) 130.4 (3.8)	0.007	45	16.55	15.58
Landisville Erie	Pupae	M	38 25	74.1 (1.8) 83.1 (2.6)	0.007	44	8.79	12.14
Landisville Erie	Pupae	F	22 29	97.8 (3.0) 106.2 (2.9)	0.049	47	8.40	8.58
Landisville Bradford	Larvae	M	38 31	84.7 (1.9) 96.8 (2.8)	0.001	54	12.11	14.28
Landisville Bradford	Larvae	F	22 38	113.8 (4.4) 131.6 (3.1)	0.002	41	17.82	15.64
Landisville Bradford	Pupae	M	38 31	74.1 (1.8) 81.1 (2.6)	0.028	55	7.01	9.44
Landisville Bradford	Pupae	F	22 38	97.8 (3.0) 108.2 (2.9)	0.016	52	10.37	10.63
Erie Bradford	Larvae	M	25 31	102.2 (3.0) 96.8 (2.8)	0.191	52	5.39	5.57
Erie Bradford	Larvae	F	29 38	130.4 (3.8) 131.6 (3.7)	0.798	57	1.27	0.92
Erie Bradford	Pupae	M	25 31	83.1 (2.6) 81.1 (2.6)	0.597	52	1.95	2.46
Erie Bradford	Pupae	F	29 38	106.2 (2.9) 108.2 (2.9)	0.632	63	1.97	1.88

* $P \geq 0.05$ are not significantly different

Table 3.3: Comparison of pupal weight of non-diapause (F1) offspring of univoltine and multivoltine ecotype established from 2005 over-wintered *O. nubilalis* population collected from central Pennsylvania.

Ecotype Compared	**Life stage compared**	**Sex**	**N**	**Mean weight in mg (SE)**	**P-Value**	**DF**	**Estimated difference (mg)**	**Weight difference (%)**
Non-diapause univoltine	Pupae	M	83	89.6 (1.2)	0.495	150	1.07	1.24
Non-diapause multivoltine			74	88.54 (0.97)				
Non-diapause univoltine	Pupae	F	90	115.4 (1.6)	0.092	159	3.47	3.03
Non-diapause multivoltine			73	118.9 (1.3)				

* $P \geq 0.05$ are not significantly different
Note: Non-diapause populations are laboratory reared.

Table 3.4: Comparison of pupal weight of 2005 post-diapausing and non-diapausing (F1) offspring of univoltine and multivoltine ecotype *O. nubilalis* population collected from central Pennsylvania.

Ecotype Compared	**Life stage compared**	**Sex**	**N**	**Mean weight in mg (SE)**	**P-Value**	**DF**	**Estimated difference (mg)**	**Weight difference (%)**
Non-diapause univoltine	Pupae	M	83	89.6 (1.2)	0.0001	119	12.46	16.21
Post-diapause univoltine			67	77.1 (1.8)				
Non-diapause univoltine	Pupae	F	90	115.4 (1.6)	0.0001	165	14.39	14.25
Post-diapause univoltine			83	101.0 (1.7)				
Non-diapause Multivoltine	Pupae	M	74	88.54 (0.97)	0.0001	75	15.24	20.79
Post-diapause Multivoltine			47	73.3 (1.7)				
Non-diapause Multivoltine	Pupae	F	73	118.9 (1.3)	0.0001	54	26.72	28.95
Post-diapause Multivoltine			41	92.2 (3.0)				

* $P \geq 0.05$ are not significantly different

Note: Non-diapause populations are laboratory reared, post-diapause population are field collected.

Table 3.5: Comparison of reproductive parameters among the eleven (A-K) *Ostrinia nubilalis* groups categorized based on the degree-day requirements for pupation after diapause termination.

Groups (Degree-day interval)	Parents mean degree-days for pupation (^oC)	Number of Parents (n)	Mean							
			Pre-oviposition period (days)	Oviposition period (days)	Post-oviposition period (days)	Females longevity (days)	Total number of egg masses	Egg mass per day	Egg per egg mass	Total number of eggs
A (50-100)	85.7	5	2.60 (0.24)	5.8 (1.39)	3.6a (0.40)	12.00 (1.34)	27.20 (3.34)	5.17 (0.55)	11.02 (1.34)	298.8 (45.86)
B (101-150)	130.5	5	3.00 (0.32)	5.6 (1.17)	2.8a (0.49)	11.60 (0.68)	24.40 (5.14)	4.40 (0.48)	16.5 (3.21)	354.2 (70.82)
C (151-200)	178.6	4	3.25 (0.95)	6.75 (1.65)	3.5a (0.65)	13.50 (2.75)	27.75 (3.50)	4.79 (0.99)	13.05 (1.20)	368. (66.90)
D (201-250)	225.4	5	3.80 (1.11)	5.2 (1.16)	1.4a (0.60)	10.40 (1.36)	26.80 (5.89)	5.19 (0.08)	13.20 (1.31)	361.4 (88.70)
E (251-300)	273.9	4	2.00 (0.41)	6.50 (1.04)	2.25a (0.75)	10.75 (0.95)	27.67 (5.62)	4.66 (0.75)	15.33 (1.30)	273.5 (67.94)
F (301-350)	327.3	5	3.00 (0.55)	6.60 (1.83)	2.20a (0.98)	11.80 (1.59)	30.40 (7.83)	4.99 (1.18)	15.15 (2.78)	396.2 (86.54)
G (351-400)	383.7	4	2.25 (0.25)	6.25 (1.97)	0.5b (0.00)	9.00 (2.12)	27.50 (9.47)	4.43 (0.56)	18.26 (1.23)	478.0 (135.52)
H (401-450)	414.7	4	2.50 (0.29)	5.25 (1.03)	2.25a (0.48)	10.00 (1.63)	27.50 (3.80)	5.77 (1.05)	12.82 (1.13)	341.0 (20.53)
I (451-500)	464.1	7	3.43 (0.65)	5.43 (0.61)	3.0a (0.69)	11.86 (0.59)	20.86 (1.60)	4.01 (0.37)	12.94 (1.30)	258.29 (11.02)
J (501-550)	529.8	4	4.25 (1.60)	6.25 (1.89)	1.75a (0.71)	12.25 (2.06)	22.00 (6.26)	3.74 (0.49)	14.38 (1.58)	342.5 (124.66)
K 551-600)	570.3	4	3.00 (0.41)	4.50 (1.04)	2.0a (0.41)	9..50 (1.71)	25.00 (6.92)	5.63 (0.64)	14.0 (0.32)	347.0 (90.01)

Notes: Means within each column followed by the same letter are not significantly different (alpha <0.05). Number in parenthesis are standard error of mean (SEM)

Table 3.6: Comparison of reproductive parameters between univoltine and multivoltine *Ostrinia nubilalis* ecotypes of the central Pennsylvania population.

Groups (Degree -day interval)	Parents mean degree-days for pupation (^{o}C)	Number of Parents (n)	Mean							
			Pre-oviposition period (days)	Oviposi-tion period (days)	Post-oviposition period (days)	Females longevity (days)	Total number of egg masses	Egg mass per day	Egg per egg mass	Total number of eggs
Multi-voltine	≤350	28	2.96 a (0.27)	6.04 a (0.53)	2.61 a (0.30)	11.64 a (0.59)	27.14 a (2.09)	4.79 a (0.29)	14.1 a (0.87)	343.54 a (28.33)
Uni-voltine	>350 - 600	23	3.13 a (0.35)	5.52 a (0.53)	2.04 a (0.32)	10.70a (0.67)	24.09 a (2.26)	4.62 a (0.30)	14.3 a (0.66)	340.96 a (35.49)

Notes: Means within each column followed by the same letter are not significantly different (alpha <0.05). Number in parenthesis are standard error of mean (SEM).

Chapter 4.

Susceptibility of co-occurring univoltine and bivoltine *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) populations to Cry1Ab and Cry1F *Bacillus thuringiensis* toxin.

4.1 Introduction.

First noticed in 1917 near Boston, European corn borer, *Ostrinia nubilalis* (Hübner), was accidentally introduced into North America through the importation of broomcorn from Hungary and Italy (Smith 1920, Baker et al, 1949). When first introduced the European corn borer produced only one generation each year (Ficht 1936, Vance 1942), by the late 1930s, however, a two-generation per year European corn borer population appear in eastern and North Central States. Today, both univoltine and bivoltine races of European corn borer are recognized across the pest's geographic range, along with two sex pheromone races (Mason et al. 1996).

In central Pennsylvania, both univoltine and bivoltine ecotypes of European corn borer co-exist and the two ecotypes emerge during distinct periods of the growing season (Calvin and Song 1994). The growing season in central Pennsylvania allows enough time for two generations of the mulitvoltine to develop, hence forth-called bivoltine. Previous study (chapter 2) revealed that bivoltine and univoltine populations vary in both post and non-diapause (F_1 generation) degree day requirements. Bivoltine population required lower degree day from egg hatch to pupation than univoltine population. When offspring from each two ecotype was reared separately for multiple generations they conserved the degree-day requirement range of their parent's. Although the seasonal occurrence of univoltine and bivoltine *O. nubilalis* is asynchronous there is some overlap in central Pennsylvania allowing the potential gene exchange.

Commercial use of transgenic corn (*Zea mays* L.) that expresses the delta endotoxin produced by *Bacillus thuringiensis* Berliner to control *O. nubilalis* began in 1996 (Koziel et al. 1993, Fischhoff 1996). After its commercial release, the popularity of *Bt* corn increased in the United Sates and around the world. *Bt* corn's season long, persistent control and widespread resistance of some lepidopteron pest populations to insecticides made it a popular pest management tactic (Tabashnik 1994, Ostile et al. 1997, Pilcher et al. 2002). According to the national corn growers association, in 2005, 45 percent of field corn planted in the U.S. nationally contained a *Bt* gene.

Because *Bt* is closed to 100% effective in controlling *O. nubilalis* population and it's easy of use and low cost, grower adoption of the technology is approaching the maximum level for resistance management plans (80%) in some geographic areas. Thus, concerns have been raised about the long term viability of *Bt* corn because some insects have already developed resistance to commercial formulations of *Bacillus thuringiensis* (Mcgaughey 1985, Mcgaughey and Johnson 1992, Mcgaughey and Whalon 1992, Shelton et al. 1993, Whalon et al. 1993, Tabashnik 1994, Huang et al. 1997, Reardon et al. 2004). Resistance to *Bt* toxin has already been detected in a field population of diamondback moth, *Plutella xylostella* L. (Kirsch et al. 1988, Tabashnik et al. 1990, 1994, Kao et al. 1994). In addition, laboratory selection studies have lead to expressed resistance to *Bt* toxin by several insect species (Schnepf et al. 1998, Frutos et al. 1999, Sanchis 2000, Van Rie 2002).

Resistance development is a real threat to transgenic *Bt* corn (Fishhoff 1996). Resistant colonies of *O. nubilalis* have also been documented. *Ostrinia nubilalis* collected from northeast Kansas and Iowa (Huang et al, 1997, 1999a) were found to be

resistant. Increased resistance (162-fold) to Cry1Ac toxin was also documented after only eight generations in a population collected from Southeastern Minnesota (Bolin et al. 1999). Selected colonies of the Minnesota population demonstrated a 16-fold resistance to the Cry1Ab *Bt* toxin (Bolin et al. 1999). Low levels of resistance to Cry1Ab were seen in lab population from Nebraska (USA) and in Italy (Chaufaux et al. 2001). Extensive use of transgenic corn is likely to increase the likelihood of selection for *Bt* resistance genes in pest populations hence diminishing the viability of this valuable management tactic (Gould 1998, (Wolfenbarger and Phifer 2000Wolfenbarger et al. 2002).

In central Pennsylvania univoltine and multivoltine ecotypes differ primarily in their post-diapause development requirement which shifts the timing of the univoltine adult flight to the period between the first and second generation of the bivoltine. However, there is a slight of overlap in flights during the beginning and end of the univoltine adult flight. This period of overlap produces an opportunity for gene exchange between the ecotypes. If one ecotype is more susceptible to the toxin than the other there would be an opportunity to transfer resistance between ecotypes.

Selection for resistance can depend on several factors. For example, the number of generations produced per year by a specific pest population can influence the rate of resistance evolution. The more generation per a growing season the more opportunities for selection of a resistant genotype. Hence, the bivoltine population may have the propensity to build resistance faster than the univoltine populations. Effective monitoring programs are a vital part of implementing resistance management strategy and a successful IRM program largely depends upon the use of appropriate bioassay techniques and the establishment of susceptibility data among the ecotypes of a target species. If

resistance to a single toxin occurs in a specific ecotype, resistance management strategies such as alternating or stacking multiple gene that are unaffected by cross-resistance could be introduce (Siqueira et al. 2004).

Selection pressure for *Bt* resistance may be increasing in central Pennsylvania due to the high level of transgenic corn cultivation in the region. Resistance could evolve in either or both of the co-occurring ECB ecotypes simultaneously or in one ecotype first and then passed to the other through intermating. The relative susceptibility of different ecotypes within a geographical location and among voltine ecotypes has not been well studied. Marcon et al. 1999 found no variation in susceptibility against Cry1Ab and Cry1Ac *Bt* toxin due to the prior exposure to selection pressure among the voltine ecotypes occurred in distinct geographical locations. However, this study did not separated voltine population rather used the whole population.

In the present study, the susceptibility of univoltine and bivoltine European corn borer populations to the Cry1F and Cry1Ab endotoxins produced by *Bacillus thuringiensis* Berliner (Vaeck et al. 1987) was compared. The comparisons were made between populations of univoltine and bivoltine European corn borer collected in Central Pennsylvania. The $LC_{50,}$ $LC_{95,}$ and LC_{99} of univoltine and bivoltine ECB population were determined for Cry1F and Cry1Ab *Bt* toxin. The sub-lethal effects of these two Cry proteins on univoltine and bivoltine European corn borer population were also measured.

4.2 Materials and Methods

4.2.1 Insect Colony:

European corn borer larvae were collected (overwintering 5^{th} instar) from corn stubble in several fields at the Russell E. Larsen Agricultural Research Center near Rock Spring, Pennsylvania. Between mid-April to early-May of 2005 about 450 diapausing 5^{th} instars larvae were collected. Each larva was individually placed in a 30 ml plastic cup and then transferred to a growth chamber in the department of Entomology at Pennsylvania State University. A water soaked small cotton ball was placed in each cup to provide sufficient moisture for larval survival. The cups were kept in an environmental growth chamber (Revco model no. RI-23-555-A) maintained at 25^0C ($\pm$ 0.5^0C) and 18L: 6D photoperiod and 65$\pm$ 5 % RH. No diet was provided during the post-diapause development period. Larvae in each cup were marked with a serial number and the date of collection was recorded to calculate the exact number of degree-days that a larva experienced while in the growth chamber. Resulting pupae were left in the growth chamber until adult emergence. Adults were put into another growth chamber for mating and oviposition.

Degree-days were calculated following the methods described by Arnold (1959) using 12.5^0C as the base threshold of *O. nubilalis* development (Calvin et al. 1991). To calculate the degree-days that accumulated in the field prior to the date a larva was collected, temperature data was spatially interpolated to a 1 square km resolution for the geographic coordinates that larvae were collected (Russo et. al. 1987). The total number of accumulated degree-days experienced by a larva was calculated by adding the number of degree-days accumulated from January 1 to the date a larva was collected in the field

to the number of degree-days accumulated after collection and placement in the growth chamber.

When a larva is collected in the field, it is not possible to determine with 100% certainty whether it is univoltine or bivoltine. To segregate univoltine and bivoltine from the collected population, we used the method described by Calvin and Song (1994). Univoltine *O. nubilalis* required >350 degree days to reach pupation in the spring, while bivoltine required ≤ 350 degree-days. A 150 degree-day interval was created as a buffer to minimize misclassification between univoltine and bivoltine because of overlapping degree day requirement. In the present experiment adults resulting within 50-300 DD pupation period were designated bivoltine and adults resulting within ≥451 DD pupation periods were designated univoltine. Univoltine and bivoltine adults were transferred into two separate wooden cages. The cages were misted daily to ensure adequate moisture, and the adults were provided honey solution to maximize fecundity and longevity (Leahy and Andow 1994). Egg masses from the univoltine and bivoltine field collected parents (F1) were placed in a screen-topped Mason jar with a moist towel inside and incubated until larvae hatched. Thus, univoltine and bivoltine colonies were established in the laboratory. The first generation (F1) neonate larvae hatched from field-collected parents' eggs were used in the Cry1F and Cry1Ab *Bt* endotoxin bioassay.

4.2.2 Bioassay procedure:

The experiment was conducted by exposing individual European corn borer neonate larvae to the surface of a single well of wheat-germ-based artificial diet (Southland products inc, Lake village, Arkansas) in a 128 cell tray (C-D international, Pitman, NJ). Each well was 16 mm in diameter and 16 mm deep. Approximately, 2 ml of

artificial diet was poured in each cell. After pouring the diet, trays were kept at room temperature for diet solidification and then stored at $4^{o}C$ up to seven days until used. The surface of the diet in each well was overlaid with a single concentrations of the Cry1F or Cry1AB toxin (Keaster and Harrendo 1965, Siegfried et al. 1995). The quantity of toxin used per well was determined by calculating the coverage of toxin per square centimeter of diet. Stock solutions were prepared for both Cry1F and Cry1Ab. A serial dilution of 0, 1, 3, 10, 30, 100, 300, and 1000 ng/cm^2 was prepared for Cry1F bioassay. For Cry1Ab bioassays, concentrations of 0, 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, and 4.0ng/cm^2 were prepared. To obtain uniform spreading, all dilutions were prepared by using a 0.05% Triton X-100 non-ionic detergent solution (Morris 1988).

In each well, exactly 100µl of the appropriate concentration of toxin was transferred onto the surface of the diet using a micropipette. Following transfer, the trays were repeatedly tilted in all directions to make sure the entire surface of the diet was covered with the *Bt* toxin. The micropipette tube was used to remove bubbles and to overlay any uncovered area. Trays were then allowed to air dry for an hour to evaporate excess water that could suffocate the neonate larva. After about one and half hours, a fine pointed paintbrush was used to transfer single neonate (with in 24hrs after hatching) larvae into each of the 128 wells. For each of eight concentrations there were four randomized replicates of 32 larva (n = 128) used. Two hundred and fifty six larvae were used for a single replication of eight treatments. A total of 1024-neonate larvae were used for four replications of each population (univoltine or bivoltine) to expose them to eight concentrations of each toxin. The diet for the control (0.0 ng/m^2) larvae was treated with only 0.05% Triton X-100 solution. Infested trays were then sealed with a vented plastic

cover (Bio-CA-16, C-D international, Pitman, NJ) and then placed at room temperature ($25\pm2^{o}C$) and 24:0 (L:D) photoperiod. Larval mortality and development stage (instar) were assessed on the 7^{th} day after being transferred onto treated diet. A larva was considered dead if it failed to move when touched or severely stunted (weight < 0.1 mg) and had not grown beyond 1^{st} instar. Surviving larvae were weighed after 7 days exposure to the toxin. Individual larval weight was then transformed into percentage of growth inhibition relative to mean weight gain in the control treatments (Siegfried et al. 2000). Based on the availability of the surviving larvae, as many as ten surviving larva were individually weighed from each of four replications from all concentration.

4.2.3 Data Analysis:

SAS Probit Analysis (SAS Institute 2004) was used to generate lethal concentration (LC_{50}) and discriminating dose (LC_{95} and LC_{99}) for univoltine and bivoltine neonate larva exposed to the Cry1F and Cry1Ab toxins. Analysis of variance (ANOVA) was performed and mean $LC_{50,}$ LC_{95} and LC_{99} were compared using Tukey and Dunnet's test (SAS Institute 2004). The general linear model procedure of SAS was used for mortality analysis and the significant level was set at $p = 0.05$, with a 95% confidence interval. Influence of toxin concentration on percentage mortality was assessed by using the Proc Mixed procedure (SAS Institute 2004) to compare the mortality rate across the concentrations. Percent mortality data were transformed by using an Arcsine-square-root transformation and ANOVA was conducted to compare the mortality between univoltine and bivoltine larva exposed to *Bt* toxin. Growth inhibition due to sublethal exposure of Cry1F and Cry1Ab toxin were assessed. Surviving larval weights were transformed by logarithmic base 10 to get homogeneity of variance and

normality. Comparison of the growth inhibition rate across concentrations was assessed by using ANOVA (SAS institute 2004) and Mixed procedure on toxin concentrations, replications, and sub lethal effects.

4.3 Results and Discussion:

4.3.1 Mortality estimate:

Mortality percentages were calculated for both univoltine and bivoltine European corn borer larvae exposed to each concentration of Cry1Ab *Bt* toxin. Figure 4.1 showed the mortality rate (log transformed) of *O. nubilalis* larvae after exposure to each concentration of Cry1Ab toxin. For the univoltine population larvae exposed to the 0.063, 0.125, and 0.250 ng/cm^2 concentrations, 39.4%, 40.6%, and 53.0% mortality was measured, respectively. At the dose 0.50 ng/cm^2 mortality was 80.5%. At the highest three doses (i.e. 1.0, 2.0, and 4.0 ng/cm^2) mortality ranged between 82 -96%. When the bivoltine population was exposed to 0.063, 0.125, 0.25 ng/cm^{2}of toxin mortalities of 43.0, 56.2, and 57.8% were measured, respectively. At the dose 0.25 ng/cm^2 57.8% mortality was observed, which was 22.7% lower than that of the univoltine population. At 0.50 ng/cm^2 85.1% mortality was observed in bivoltine population compared to 80.5% mortality for the univoltine population. At the highest three doses mortality ranged from 85-100%.

Univoltine and bivoltine larvae expressed similar mortality rate at each concentration of Cry1F. Figure 4.2 showed the mortality rate (log transformed) of *O. nubilalis* larvae after exposure to each concentration of Cry1F toxin. Univoltine larvae exposed to 1.0, 3.0, 10.0, and 30.0 ng/cm^2 expressed 13.3, 18.7, 34.0, and 54.7%

mortality respectively. At the dose 100 ng/cm^2 81.2% larval mortality was measured. Between 94 – 100%, larval mortality was observed at the highest two doses (i.e. 300 and 1000 ng/cm^2 toxin). Similarly, the bivoltine population exposed to1.0, 3.0, 10.0, and 30.0 ng/cm^2 of Cry1F toxin expressed 16.9, 18.7, 44.5, and 57.9% mortality, respectively. Larvae exposed to the 100 ng/cm^2 dose expressed 77.2% mortality. At the highest two doses, larval mortality ranged between 96 -100% when exposed to Cry1F *Bt* toxin.

When exposed to Cry1Ab toxin percentage mortality of bivoltine ecotype was siginificantly higher than univoltine ($F = 6.54$; $df = 1$; $Pr > 0.014$). However, there was no significant difference in over all dose-response pattern (interaction effect) when neonates were exposed to the Cry1Ab toxin ($F = 0.85$; $df = 7$; $Pr > 0.552$). For the univoltine and bivoltine colonies exposed to the Cry1F toxin there were no significant differences observed ($F = 1.30$; $df = 1$; $Pr > 0.259$). Similar analysis showed that the dose-response pattern (interaction effect) was similar ($F = 0.38$; $df = 7$; $Pr > 0.907$ for univoltine and bivoltine neonates exposed to Cry1F toxin (Table 4.1).

Table4.2 compares individual larval response to the eight dose concentrations used to determine the susceptibility of the two ecotypes exposed to Cry1Ab and Cry1F *Bt* toxins. When exposed to Cry1Ab *Bt* toxin, except for the 2.0 ng/cm^2 concentration ($F = 4.46$; $df = 1$; $Pr > 0.040$), the dose-response comparisons at all the eight concentrations were not significantly different between univoltine and bivoltine ecotypes.. None of the eight concentrations used for Cry1F showed any susceptibility difference between univoltine and bivoltine. Small variations in response to 2.0 ng/cm^2 Cry1Ab toxin might be the consequences of the natural variation in the European corn borer population.

4.3.2 Toxin Response:

Susceptibility data for univoltine and bivoltine *O. nubilalis* populations exposed to Cry1F and Cry1Ab toxins are presented in table 4.3. The lethal concentration (L_C) values for Cry1F expressed in table 4.3 are based on the active ingredient of the toxin (13.7% a.i.). The LC_{50}, value for the univoltine population exposed to Cry1F *Bt* toxin was 4.60 ng/cm^2, similarly, the LC_{50} value for the bivoltine population exposed to same toxin was 4.08 ng/cm^2. The LC_{50} dose for univoltine and bivoltine populations was not significantly different (Pr > F=0.58) (table 4.4). The LC_{95} values for univoltine and bivoltine populations exposed to Cry1F *Bt* toxin were 15.4 and 14.9 ng/cm^2 respectively, and the LC_{99} for univoltine and bivoltine populations exposed to the same toxin were 19.9 and 19.4 ng/cm^2 respectively. The LC_{95} (Pr > F = 0.89) and LC_{99} (Pr > F = 0.927) were not significantly different for univoltine and bivoltine populations exposed to Cry1F *Bt* toxin (Table 4.4).

However, there were response differences when exposed to Cry1Ab. The LC_{50} value for the univoltine population exposed to Cry1Ab was 0.35 ng/cm^2, while the LC_{50} for bivoltine population exposed to Cry1Ab was 0.21ng/cm^2. The LC_{95} values for univoltine and bivoltine population exposed to Cry1Ab were 2.66 and 1.41 ng/cm^2, respectively. The LC_{99} values for univoltine and bivoltine populations exposed to Cry1Ab were estimated to be 3.62 and 1.90 ng/cm^2, respectively. Although, variation in susceptibility to Cry1Ab was observed between ecotype populations, the magnitude of these differences was only 1.5- to 2-fold. Tukey's Studentized Range test and Dunnett's test suggested that the differences in lethal doses were not significantly different (Pr >

0.186, 0.896, and 0.083 was for the LC_{50}, LC_{95}, and LC_{99} values, respectively) (Table 4.4).

Previous studies also found a similar magnitude of variation, but never considered it as the basis of genetic variation among the populations or potential resistance development, but rather considered it as natural population variation. A Cry1Ab and Cry1Ac baseline susceptibility test on European corn borer populations collected from several locations in the U.S. showed 2- to 3- fold differences in LC_{50} and in EC_{50} and a 2- to 6- fold differences were found in LC_{95} and EC_{95} (Marcon et al. 1999). This magnitude in susceptibility was also observed across generations within a particular European corn borer population. Marcon et al. considered this interpopulation variation in susceptibility might be due to the higher fitness of some neonates gained through the nutritional quality of the eggs produced by the parental female. Stone and Sims (1993) found 16-fold variation in susceptibility among corn ear worm (*Helicoverpa zea*) populations and 4-fold susceptibility in tobacco budworm (*Heliothis virescens*) populations exposed to the Cry1Ac *Bt* toxin and considered those interpopulation variation as non-genetic or by sampling error from a small representation of a larger univoltine or bivoltine population. Significant variation in *Bt* susceptibility was observed in three populations of Gypsy moth (*Lymantria dispar*), but this variation was mostly noticed among the siblings within a family rather than across families (Rossiter et al. 1990). Variation in susceptibility between ECB populations also was found due to the use of different bioassay technique (Siegfried et al. 1995). The bioassay method used in this study was highly recognized by the current researcher.

The small variation observed in our study might be the result of natural variation in parental vigor which can be influenced by host plant (*Zea mays*) nutritional status due to the season long emergence pattern of co-occurring ecotypes in the Rock Spring area. Conversely, it might be the tip of a potential resistance development due to high use of *Bt* corn in the area. In this study, exposed larvae were selected from a large parent population collected from a location where corn was cultivated continually for the last five years. Moreover, exposed neonates of *O. nubillais* were collected from the whole range of the population's pupation period (i.e. 75 DD to ending at 700 DD). So, the issue of sampling error was addressed well in this study. Further research with distinct univoltine and multivoltine population collected from several locations may confirm the potentiality of the resistance development between univoltine and bivoltine population.

4.3.3 Growth Inhibition:

Growth inhibitions due to sublethal effects are more sensitive than mortality for establishing an integrated resistance management (IRM) program (Gould 1998, Onstad and Gould 1998). A comparison was done between univoltine and bivoltine larval weight reductions due to the sublethal effects. Figure 4.6 showed the growth inhibition rate (log transformed) of *O. nubilalis* larvae after exposure to each concentration of Cry1Ab toxin. Univoltine and bivoltine larvae had a strong anti-feedant effect (identified by no feeding mark on diet surface after 7 days exposure to the toxin treated diet) and a significant growth stunt in individuals was able to survive after exposure to the Cry1Ab *Bt* toxin. Larva exposed to similar or lower concentrations than the LC_{50} of Cry1Ab (i.e. 0.0625, 0.125 and 0.25 ng/cm^2) expressed 78.3, 86.4, 90.1 percent and 71.1, 71.0, 78.2 percent

growth inhibition for univoltine and bivoltine population, respectively, compared to the untreated control (Figure 4.3). If growth inhibition is compared for the lower three concentrations (sublethal doses) of Cry1Ab, there was a significant over-all difference ($F = 8.51$; $df = 1$; $Pr < 0.003$) in growth inhibition between univoltine and bivoltine. However, the growth inhibition pattern was not significantly different across those three doses ($F = 0.12$; $df = 2$; $Pr > 0.89$) (Table 4.5). On the other hand, larvae exposed to the higher four concentrations (4.0, 2.0, 1.0, and 0.50 ng/cm^2) of Cry1Ab, showed no significant over-all difference ($F = 1.55$; $df = 1$; $Pr < 0.214$) in growth inhibition between the two ecotypes and the growth inhibition pattern was found to be not significantly different across those four doses ($F = 0.53$; $df = 2$; $Pr > 0.663$) (Table 4.5). The mean weight variation in first instar larval growth at the lower or sublethal concentrations may elucidate resistance variability between ecotype populations, but when higher doses were used this resistance tendency may be masked by the mortality caused by higher doses. This is an integral part of the high dose strategy in the IRM program**.** Killing the heterozygous individuals in ECB population is the focal point of high-dose refuge strategy to avoid or slow the evolution of resistance (Tabashnik et al 1998).

Converse to Cry1Ab, univoltine larvae exposed to the four lower concentrations (sublethal doses) of Cry1F expressed 32.9, 56.0, 65.8, and 89.3 percent growth inhibition. Similarly, bivoltine larvae exposed to the four lower concentrations of Cry1F expressed 34.0, 34.4, 61.2, and 73.1 percent growth inhibition (Figure 4.4). Figure 4.5 showed the growth inhibition rate (log transformed) of *O. nubilalis* larvae after exposure to each concentration of Cry1F toxin. When we compared univoltine and bivoltine growth inhibition at the lower four concentrations of Cry1F very closer to significant difference

was observed (F = 3.47; df = 1; Pr > 0.063), However, the growth inhibition pattern was significantly different (F = 4.25; df = 3; Pr > 0.0058). That means at least one of those four lower concentrations had a significant growth inhibition difference between univoltine and bivoltine. In this case, a 30 ng/cm^2 concentration is the discriminating growth inhibition dose between univoltine and bivoltine ecotypes (Table 4.7). On the other hand, a growth inhibition of larvae exposed to the higher three concentrations of Cry1F there was a significant over-all difference (F = 8.04; df = 1; Pr < 0.005) and the growth inhibition pattern was also found to be significantly different (F = 3.63; df = 2; Pr > 0.029) (Table 4.5). Although, sublethal dose effects were measurable under laboratory conditions, they may not show up under field conditions because of the significantly higher concentration levels expressed in corn plants. Thus, the laboratory measured survival rates observed at reduced concentrations may have little relevance to resistance evolution under field conditions.

When exposed to Cry1F the LC_{50} ratio for univoltine and bivoltine populations was almost the same (4.60 and 4.08 ng/cm^2 respectively) where as the EC_{50} ratios were 5.6 times higher for bivoltine population than univoltine (Table 4.3). This result suggests that the Cry1F toxin has a much greater impact on the univoltine European corn borer population's growth and development than the bivoltine population's growth at concentrations below the LC_{50} (i.e.sublethal concentrations). Similar growth inhibition analysis for univoltine and bivoltine populations exposed to the lower concentrations of the Cry1Ab toxin generated a negative EC_{50} value means that the lowest dose of Cry1Ab (0.0625ng/cm^2) itself reduced more than 50% of the survived larval weight than the control. Thus, even though the LC_{50} value for univoltine and bivoltine (0.35 and

0.21ng/cm^2) populations was 5- and 3-fold higher, respectively, than the lowest concentration (0.0625ng/cm2), the lowest concentration had a much higher impact on both univoltine and bivoltine population when the total effect of mortality and reduced fitness due to growth inhibition, are taken into account. A 78% and 71% growth inhibition at the lowest concentration for univoltine and bivoltine population was observed, respectively (Figure 4.3). At this level of growth inhibition it was assumed that none of the surviving larvae would be able to complete their life cycle to produce future generations. In summary, both univoltine and bivoltine ecotypes larval growth was reduced incrementally when exposed to higher doses of either Cry1Ab or Cry1F.

Analysis of variance on each of the eight concentrations suggested no differences on overall larval growth between univoltine and bivoltine population exposed to Cry1Ab *Bt* toxin ($F = 0.23$; $df = 1$, $P > 0.628$). The interaction analysis between ecotype and toxin concentration showed that growth inhibition was similar between univoltine and bivoltine populations across the doses of Cry1Ab ($F = 1.45$; $df = 7$; $Pr > 0.183$) (Table 4.6). A concentration wise comparison between univoltine and bivoltine ecotypes indicated that except at 0.25 ng/cm^2 there were no significant differences ($Pr > 0.05$) in larval growth after seven days of exposure to Cry1Ab (Table 4.7). Similar analysis for Cry1F showed that there was variation in overall growth between univoltine ($F = 10.71$, $df = 7$, $Pr > 0.001$) and bivoltine (Table 4.6). Concentration wise comparison between univoltine and bivoltine ecotypes indicated that there were differences in larval growth when exposed to 30 and 100 ng/cm^2 toxin concentrations ($Pr < 0.001$)(Table 4.7). These variations in larval growth might arose from the sublethal affect of Cry1F *Bt* toxin.

The consequences of these sublethal effects were not evaluated in this experiment, but it may have importance in *Bt* refuge management due to the potential variation in the developmental time requirement for adult emergence in the field population exposed to these *Bt* toxins and the refuge population. Sublethal effects of *Bt* toxins on the fitness of insect life history were reported in several other studies. After exposure to the Cry1Ab toxin, Siegfried et al. (2001) found 10% higher survival rates and lower parasitism rates in the F1 progeny of the post diapause *O. nubilalis* collected from a *Bt* hybrid field. Storer et al. (2001) found 6-10 days delay in pupation and adult eclosion by feeding *Heliocoverpa zea* on *Bt* corn ears. Extended developmental period (1.8-4.5 days for pupation and 4-8 days for adult eclosion) in Colorado potato beetle after ingestion of sublethal doses of *B. thuringiensis* (Costa et al. 2000, Nault et al. 2000). In the Mediterranean basin, Dipel DF treated offspring from post-diapause *Sesamia nonagrioides*, collected from *Bt* field, expressed higher mortality, longer developmental time, extra molts, and higher sensitivity to critical day length for diapause induction than the untreated larvae (Eizaguirre et al. 2005). For ECB in central Pennsylvania, a developmental delay caused by a sublethal dose exposure, may generate an adult emergence asynchrony between the *Bt* and non-*Bt* (refuge) corn host, disrupting the resistance management strategy.

4.4 References:

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Figure 4.1: Transformed mortality of univoltine and bivoltine European corn borer after 7 days exposure to Cry1Ab *Bt*. Toxin.

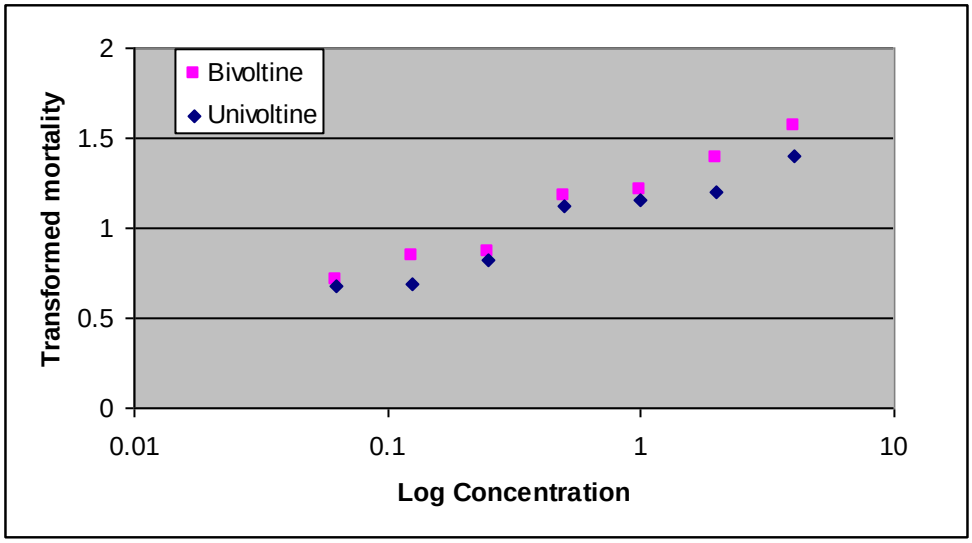


Figure 4.2: Transformed mortality of univoltine and bivoltine European corn borer after 7 days exposure to Cry1F *Bt*. Toxin

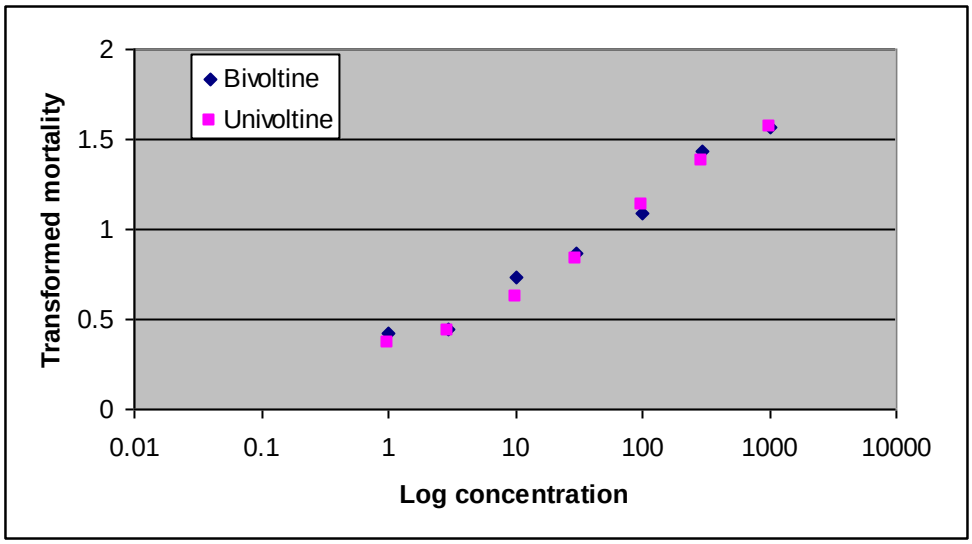


Figure 4.3: Percentage of growth inhibition of univoltine (A) and bivoltine (B) European corn borer neonate larvae exposed to different concentrations of the Cry1Ab *Bt.* toxin.

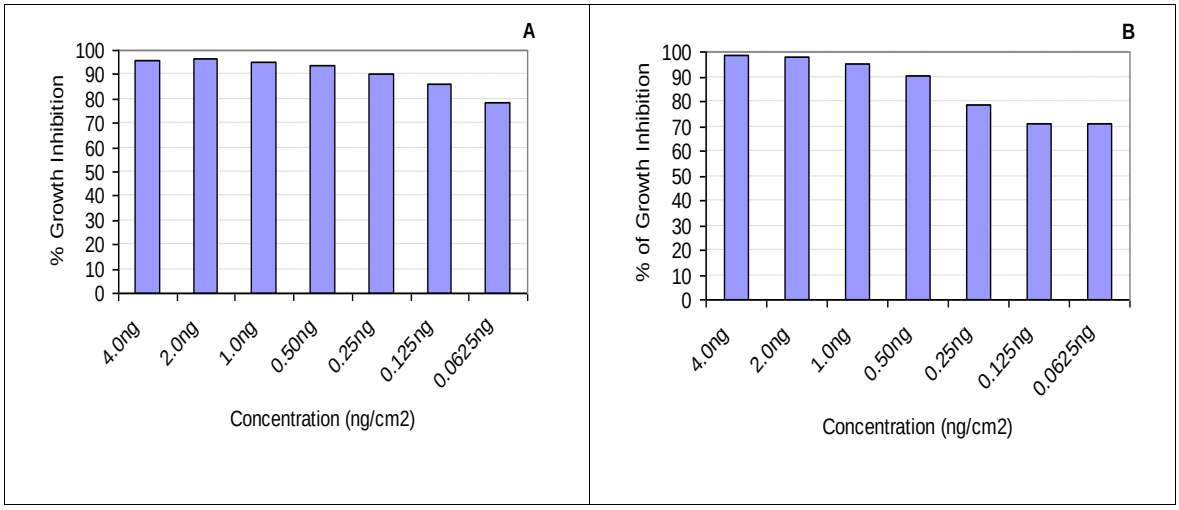


Figure 4.4: Percentage of growth inhibition of univoltine (A) and bivoltine (B) European corn borer neonate larvae exposed to different concentrations of the Cry1F *Bt* toxin.

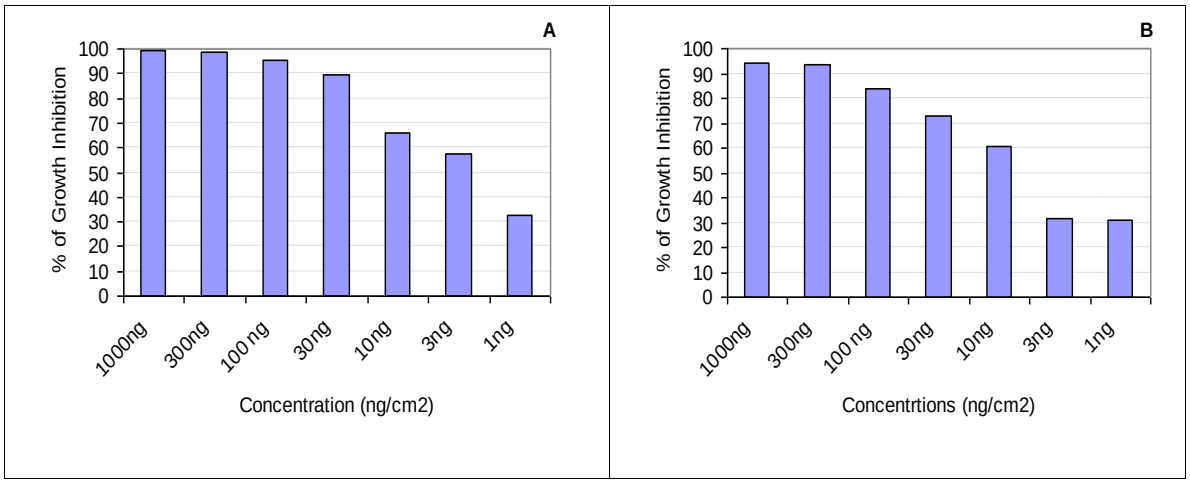


Figure 4.5: First instar European corn borer larval growth inhibition after 7 days of exposure to different concentration of Cry1F *Bt* toxin

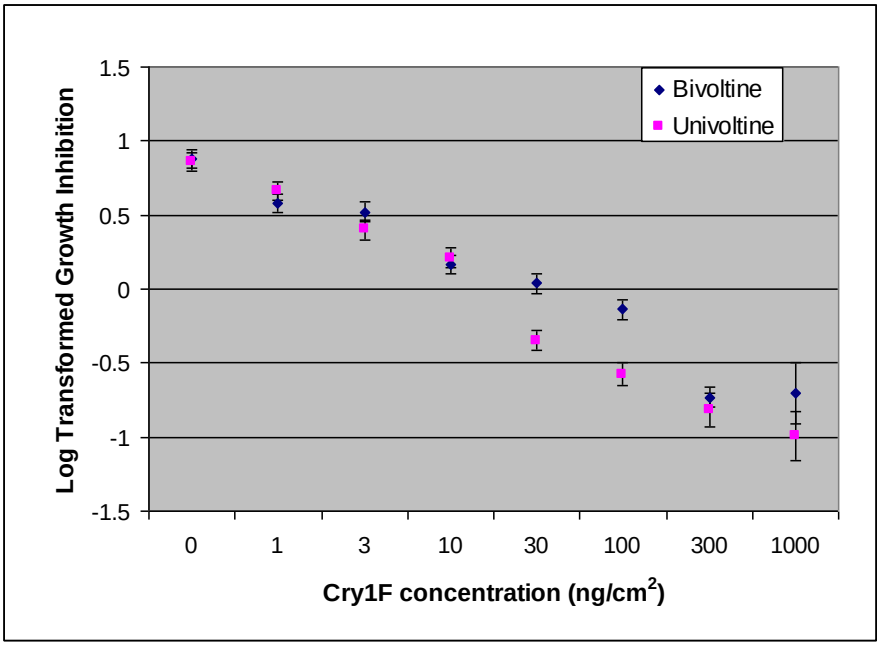


Figure 4.6: First instar European corn borer larval growth inhibition after 7 days of exposure to different concentration of Cry1Ab *Bt* toxin.

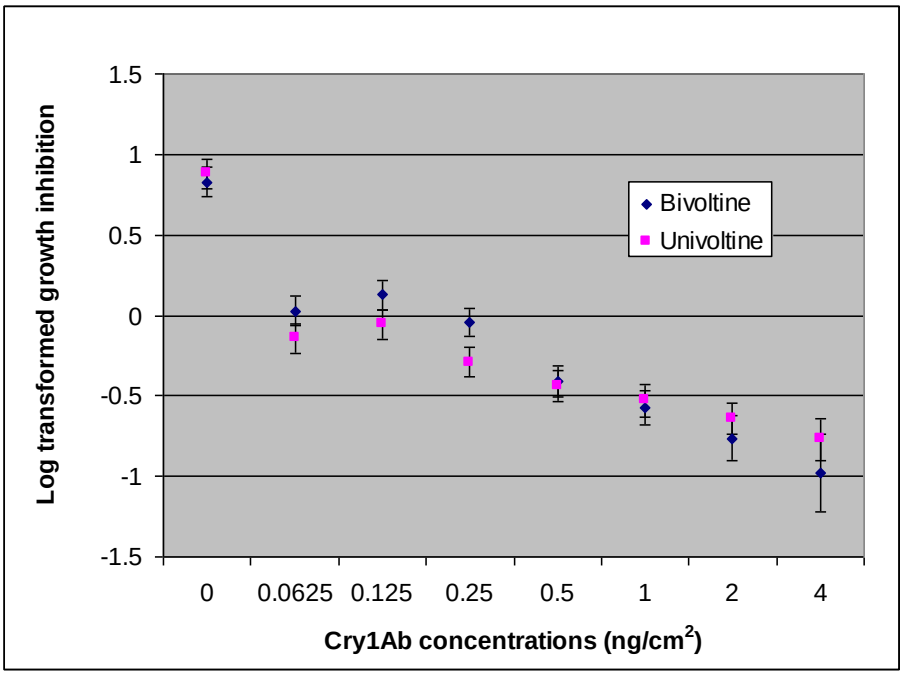


Table 4.1: ANOVA comparing overall dose response for Cry1Ab and Cry1F Arcsine-square-root transformed mortality between univoltine and bivoltine European corn borer population.

Toxin	**Effect**	**DF**	**F Value**	**Pr > F**
Cry1Ab	Ecotypes	1	6.54	0.0140
	Concentration	7	78.84	<.0001
	Ecotype*Concentration	7	0.85	0.5526
Cry1F	Ecotypes	1	1.30	0.259
	Concentration	7	178.26	<0.0001
	Ecotype*Concentration	7	0.38	0.9074

Table 4.2: ANOVA comparing individual dose response for Cry1Ab and Cry1F Arcsine-square-root transformed mortality between univoltine and bivoltine European corn borer population.

***Bt.* Toxin**	**Effect**	**Concentration (ng/cm^2)**	**DF**	**F Value**	**Pr > F**
Cry1Ab	Ecotypes* Conc	0	1	0.30	0.585
	Ecotypes* Conc	0.0625	1	0.11	0.743
	Ecotypes* Conc	0.125	1	3.08	0.086
	Ecotypes* Conc	0.25	1	0.18	0.677
	Ecotypes* Conc	0.50	1	0.44	0.511
	Ecotypes* Conc	1.0	1	0.39	0.533
	Ecotypes* Conc	2.0	1	4.46	0.040
	Ecotypes* Conc	4.0	1	3.54	0.066
Cry1F	Ecotypes* Conc	0	1	0.02	0.883
	Ecotypes* Conc	1	1	0.52	0.473
	Ecotypes* Conc	3	1	0.02	0.896
	Ecotypes* Conc	10	1	2.35	0.132
	Ecotypes* Conc	30	1	0.21	0.652
	Ecotypes* Conc	100	1	0.28	0.601
	Ecotypes* Conc	300	1	0.59	0.446
	Ecotypes* Conc	1000	1	0.00	1.000

Table 4.3: Susceptibility of European corn borer exposed to the Cry1F and Cry1Ab protein from *B. thuringiensis* as measured by growth inhibition and mortality.

***Bt* toxin**	**Populations**	**Parental population**	**N**	**EC_{50} ng/cm^2 (95% CI)**	**Slope ± SE**	**LC_{50} ng/cm^2 (95% CI)**	**LC_{95} ng/cm^2 (95% CI)**	**LC_{99} ng/cm^2 (95% CI)**	**X^2**	**df**
Cry1Ab	Univoltine, PA	172	1022	-0.27	0.71 ± 0.05	0.35 (0.22-0.47)	2.66 (2.35 -3.0)	3.62 (3.18- 4.23)	147.17	1
	Bivoltine, PA	196	1020	-0.04	1.37 ± 0.11	0.21 (0.14 - 0.28)	1.41 (1.24 – 1.63)	1.90 (1.67 – 2.22)	142.31	1
Cry1F	Univoltine, PA	182	1022	0.10 (0.00-1.97)	0.01 ± 0.0008	4.60 (3.93-5.38)	15.41 (13.56-17.91)	19.89 (17.46 -23.18)	159.38	1
	Bivoltine, PA	178	1002	0.56 (0.00-2.90)	0.01 ± 0.0009	4.08 (3.42-4.48)	14.89 (12.98 - 17.53)	19.37 (16.84 -22.89)	137.81	1

Table 4.4: Cry1AB and Cry1F lethal dose comparison between bivoltine and univoltine populations of European corn borer at Rock spring, Pennsylvania in 2005.

Toxin(s)	Population(s)	N	Mean LD_{50} (ng / sq.cm.)	Pr > F	Mean LD_{95} (ng / sq.cm.)	Pr > F	Mean LD_{99} (ng / sq.cm.)	Pr > F
Cry1Ab	Univoltine	1022	0.35a	0.186	2.66a	0.896	3.62a	0.083
	Bivoltine	1020	0.21a		1.41a		1.90a	
Cry1F	Univoltine	1022	4.60a	0.580	15.41a	0.083	19.89a	0.927
	Bivoltine	1002	4.08a		14.89a		19.37a	

Table 4.5: ANOVA comparing overall growth inhibition of first instar univoltine and bivoltine *O. nubilalis* larva after 7 days of exposure to sublethal concentrations of Cry1Ab and Cry1F *Bt* toxin overlays on diet surface.

Toxin	**Concentrations**	**Effect**	**DF**	**F Value**	**Pr > F**
Cry1Ab	Lower 3 dose	Ecotypes	1	8.51	0.0039
		Concentration	2	2.91	<.0567
		Ecotype*Concentration	2	0.12	0.8901
	Higher 4 dose	Ecotypes	1	1.55	0.2146
		Concentration	3	6.58	0.0003
		Ecotype*Concentration	3	0.53	0.6639
Cry1F	Lower 4 dose	Ecotypes	1	3.47	0.0633
		Concentration	3	43.74	<.0001
		Ecotype*Concentration	3	4.25	0.0058
	Higher 3 dose	Ecotypes	1	8.04	0.0054
		Concentration	2	24.57	<.0001
		Ecotype*Concentration	2	3.63	0.0296

Table 4.6: ANOVA comparing overall growth inhibition of first instar univoltine and bivoltine *O. nubilalis* larva after 7 days of exposure to different concentrations of Cry1Ab and Cry1F *Bt* toxin overlays on diet surface.

Toxin	**Effect**	**DF**	**F Value**	**Pr > F**
Cry1Ab	Ecotypes	1	0.23	0.6285
	Concentration	7	73.66	<.0001
	Ecotype*Concentration	7	1.45	0.1837
Cry1F	Ecotypes	1	10.71	0.0011
	Concentration	7	113.65	<.0001
	Ecotype*Concentration	7	3.93	0.0003

Table 4.7: ANOVA comparing growth inhibition of first instar univoltine and bivoltine *O. nubilalis* larva after 7 days of exposure to individual concentrations of Cry1Ab and Cry1F *Bt* toxin overlays on diet surface.

***Bt.* Toxin**	**Effect**	**Concentration (ng/cm^2)**	**DF**	**F Value**	**Pr > F**
Cry1Ab	Ecotypes* Conc	0	1	0.26	0.6131
	Ecotypes* Conc	0.0625	1	2.68	0.1022
	Ecotypes* Conc	0.125	1	3.20	0.0742
	Ecotypes* Conc	0.25	1	5.66	0.0177
	Ecotypes* Conc	0.50	1	0.05	0.8208
	Ecotypes* Conc	1.0	1	0.14	0.7085
	Ecotypes* Conc	2.0	1	0.61	0.4347
	Ecotypes* Conc	4.0	1	0.66	0.4173
Cry1F	Ecotypes* Conc	0	1	0.05	0.8156
	Ecotypes* Conc	1	1	0.81	0.3679
	Ecotypes* Conc	3	1	1.86	0.1727
	Ecotypes* Conc	10	1	0.22	0.6363
	Ecotypes* Conc	30	1	17.88	<.0001
	Ecotypes* Conc	100	1	17.70	<.0001
	Ecotypes* Conc	300	1	0.47	0.4948
	Ecotypes* Conc	1000	1	1.19	0.2755

Chapter 5.

Can a specialist parasitoids *Macrocentrus cingulum* on *Ostrinia nubilalis* influence the ecotype or race structure of its preferred host population?

5.1 Introduction:

Macrocentrus cingulum Brischke, a specialist larval parasitoid, is one of the three major successfully established parasitoids of European corn borer, *Ostrinia nubilalis* Hübner, in the United States. During the 1930's several parasitoids were imported into the United States from Asia and Europe as potential biological control agents of this pest (Baker et al. 1949). Since, its release *M. cingulum* has expanded its range from Pennsylvania to Virginia (Mason et al. 1994). The role of *M. cingulum* as a natural enemy of *O. nubilalis* was documented by several studies (Peairs and Lilly 1975, Andreadis 1982, Romig et al. 1985, Losey et al. 1992, White and Andow 2005). *Macrocentrus cingulum* has effectively reduced *O. nubilalis* populations in the northeastern and mid Atlantic region of the United States (Sked and Calvin 2005). Surveys indicated that *M. cingulum* can parasitize up to 60% of post-diapause *O. nubilalis* population in the field (Winnie and Chiang 1984).

In central Pennsylvania and in some portions of New York and Massachusetts *Ostrinia nubilalis* (Hübner) produces a mix of univoltine and multivoltine populations. The relative mix is dependent on growing season length with univoltine populations dominating in northern cooler areas and multivoltine is more in southern or warmer regions. On regions of co-occurrence two ecotypes of *O. nubilalis* can be separated based on the post-diapause heat unit requirements for spring pupation. Post-diapause ECB larva that pupated at ≤350 degree-days have accumulated are multivoltine while ECB larva those pupated at ≥351 degree-days are designated univoltine (Calvin and Song 1994). An

important question addressed by this study i.e. does *M. cingulum* equally attack multivoltine and univoltine populations of ECB, given their asynchrony during the growing season.

The potential for Bt resistance development and the elimination of its density dependent parasitoid, *M. cingulum*, created concern for the viability of this valuable management tools (Hails 2000, Obrycki et al. 2001). Availability of alternate hosts of ECB and host shift in future can change the population ecological niche other than corn host. European corn borer developed in two different host plants e.g. maize (*Zea mays L.*) and mugwort (*Artemisia vulgaris* L.) in a sympatric location in france maintained almost absolute reproductive isolation with a possible sympatric speciation (Malausa et al. 2005) and the females showed oviposition fidelity on host plant they developed (Bethenod et al. 2005). In the event of host shift in future *M. cingulum* can play a leading role in controlling ECB in other than corn host.

Until the introduction of Bt-corn technology, management of ECB relied on multiple tactics that included synthetic insecticides, cultural methods and biological control agents. The extremely high level of ECB control using Bt-corn, however, has eliminated other management tactics and jeopardizing the survival of specialized biological control agents such as *M. cingulum*. As long as Bt-corn technology remains effective the potential extinction of *M. cingulum* would be insignificant but in the event of ECB become resistant to the Bt-toxin or shifted to another host plant and *M. cingulum* is extinct the pest population could explode causing significant crop losses in the future.

The life cycle of *M. cingulum* has two phases of development. In the first phase, adult *M. cingulum* oviposite a single egg preferentially in the body of the third to fifth

instar of *O. nubilalis* larva (Parker 1930, Bruck and Lewis 1998). The egg then undergoes polyembryonic development to produce several eggs. During the internal development phase, the parasitoid's polyembryonic larvae hatch out and complete their third instar inside a host larva. No parasitoid larval development occurs until the host larvae have developed well into the fifth instar (Dittrick and Chiang 1982). After feeding internally, fourth instar *M. cingulum* larvae emerge from the body of the host and feed ectoparasitically consuming the entire host within 24 hours except the head capsule. The parasitoid larva then pupated and emerges as adults. Adult emergence of *M. cingulum* synchronized well with adult emergence *O. nubilalis* (Udayagiri et al. 1997, Bruck and Lewis 1998). However, adult *M. cingulum* emergence does not match its preferred host life stages. This asynchrony is correlated by an adult longevity of ≈17.6 days (Parker 1930) which provides necessary time to locate preferred host life stage (Quicke 1997, Sked and Calvin 2005).

Several studies were conducted to understand the biology, geographical distribution, and synchronization with host (Parker 1930, Peairs and Lilly 1975, Andreadis 1982, Winnie and Chiang 1984, Losey et al. 1992, Mason et al. 1994, Sked and Calvin 2005). In this study, we investigated the impact of *M. cingulum* on populations of *O. nubilalis* in temporally separated co-occurring univoltine and multivoltine individuals over four years at Rock spring, PA (2002, 2003, 2004, and 2005). In 2005, we also investigated the impact of this parasitoid at three additional spatially isolated locations in Pennsylvania. We observed variable sex ratios in post-diapause multivoltine and univoltine ecotype population. Our study also suggested a differential preference by *M. cingulum* towards male and female *O. nubilalis* larval host

thus gave hints for possible presence of cues generate differently by the male and female host larva. Understanding the impact of *M. cingulum* on the voltine ecotypes can further explore the effectiveness of this very important biological control agent. Furthermore, it may reinforce the necessity for refuge ECB population to minimize potential resistant populations due to the increased Bt-corn hybrid cultivation.

5.2 Materials and Methods:

Post-diapuse *O. nubilalis* fifth instar larva were collected from corn stubble in Central Pennsylvania ($40^{o}42`$ N $77^{o}57`$ W, elevation 1225 ft), a region of co-occurring univoltine and multivoltine ecotypes. Collections were made in 2002 (n = 127), 2003 (n = 449), 2004 (n = 359), and 2005 (n = 422) during the month of early April. In 2005 over-wintering larva were collected from three additional locations of Pennsylvania; Landisville in Lancaster county ($40^{o}12`$ N $76^{o}43`$ W, elevation 360 ft) south-east PA, a region dominated by the multivoltine ecotype, Erie ($42^{o}01`$ N $80^{o}12`$ W, elevation 837 ft) north-west PA, a region dominated by the univoltine ecotype, Towanda in Bradford county ($41^{o}48`$ N $76^{o}28`$ W, elevation 759 ft) north PA, a region typically dominated by the univoltine ecotype. The Number of post-diapause larva collected was 117, 75, and 85, respectively, from Lancaster, Erie and Bradford counties. After collection larvae were transferred to the laboratory and weighed. After weighing larvae place in an environmental chambers and reared at $25^{o}C$, 18:6 (L:D), and > 65% relative humidity. Larvae were monitored daily for pupation, parasitoid emergence or death. The *O. nubilalis* and *M. cingulum* pupation, parasitism, or deaths were recorded. Degree-days to key *O. nubilalis* and *M. cingulum* events were calculated using a $12.5^{o}C$ developmental threshold temperature (Calvin and Song 1994). Degree-day data for specific regions of

collection was provided by ZedX Corporation (Bellefonte, PA). ZedX spatially interpolated degree-days for the specific geographic coordinates by weighting temperature from the three nearest adjacent weather stations (Russo et al. 1987). This spatial interpolation temperature was at 1-km^2 resolutions with ±0.5^oC accuracy.

Degree-days were calculated by adding the daily maximum temperature and minimum temperature dividing by two and then subtracting the base threshold (12.5^oC) temperature (Arnold 1959). If the calculated degree-day accumulation per day was less then 1 degree-day, zero degree-days were recorded for that day. If the maximum temperature was higher and minimum temperature was lower than the base threshold temperature then degree-days were calculated by taking the (maximum – base threshold temperature) / 2. The total number of degree-days accumulated from January 1 to pupation of an individual larva was calculated by adding together degree-days accumulated while the larva was in the field and while in the laboratory growth chamber (Revco model no. RI-23-555-A, SPX Corporation, Ashville, NC). Degree day data were then entered into an Excel^{TM} spreadsheet for mathematical calculation and statistical analysis. Mathematical models were constructed to show the relationship between seasonal degree-day accumulations and the proportion of the population entered into pupal stage of *M. cingulum* and *O. nubilalis*. Percent parasitism over the post-diapause population was estimated for each year and location.

The pupation period for post-diapause *O. nubilalis* populations was divided into 50 degree-day intervals starting from 1 and ending at 1300 degree-days. The proportion of *M. cingulum* parasitism and the number of male and female *O. nubilalis* emerging in a given 50 degree-day group was calculated.

To determine sex ratio in the absence of parasitoid pressure non-diapause F_1 progenies of 2003, 2004, and 2005 univoltine and multivoltine ECB was reared in environmental chambers under the same environmental conditions as their parents experienced in the laboratory. Larval weight difference between male, female and parasitize larva were analyzed and means were separated by t-test. Regression analysis was done to establish relationships between *M. cingulum* parasitize larval weight and number of adult parasitoid emerged.

5.3 Results:

5.3.1 *Temporal variation in Central Pennsylvania:*

Spring pupation of *O. nubilalis* populations began at around 75 degree-days and ended between 650 to 1300 degree-days across years (2002 to 2005) in Central Pennsylvania. Spring pupation for *M. cingulum* began at around 125 Degree-days each year and ended at around 400 degree-days (Figure 5.1). Median pupation (50% completion) varied from 288 to 400 for *O. nubilalis* and from 210 to 240 degree-days for *M. cingulum* over years. The mean and standard errors for *O. nubilalis* and *M. cingulum* peak spring pupation periods were 353.14 $\pm$ 35.63 and 227.48 $\pm$6.44 degree-days, respectively. Figure 5.1 illustrates how the *M. cingulum* pupation period appears to be primarily synchronized with the multivoltine portion of the *O. nubilalis* spring pupation period (i.e. from 75 to 350 degree-days).

The percentage of *O. nubilalis* parasitism by *M. cingulum* varied across years in Central Pennsylvania (Table 5.1). Across years there was a significant and positive relationship between the percentage of the *O. nubilalis* population that is multivoltine and the percentage of the total population (multivoltine plus univoltine) parasitized by *M.*

cingulum (% parasitism = -2.61 + 0.483 * % of *O. nubilalis* population that is multivoltine) (R^2 = 0.644; Pr = 0.048) (Figure 5.2a). However, when the percentage of the total population that is multivoltine was regressed against the percentage of the multivoltine population parasitized there so no significant relationship (R^2 = 0.181; Pr = 0.411) (Figure 5.2b). This analysis suggests that the percentage of the multivoltine population parasitized by *M. cingulum* is not dependent on the percentage of the overall *O. nubilalis* population that is multivoltine. It emphasizes that *M. cingulum* is in fact synchronized with the multivoltine portion of the overall *O. nubilalis* population and differentially impacts the multivoltine population relative to the univoltine population.

5.3.2 *Spatial variation across Pennsylvania:*

To further investigate the relationship of *M. cingulum* and multivoltine *O. nubilalis* populations, in 2005 a population of overwintering larvae were collected in Lancaster (Landisville, PA), Bradford (Towanda, PA) and Erie (McKean, PA) Counties, Pennsylvania. Figure 5.3 shows the spring pupation periods for *O. nubilalis* populations collected in each county. The Lancaster county location (Landisville) spring pupation period of *O. nubilalis* began at around 18.0 degree-days and ended at around 405 degree-days, thus this population was primarily multivoltine (95% multivoltine and 5% univoltine). At Landisville, the *M. cingulum* population began spring pupation at around 143.3 degree-days and ended at around 330.8 degree-days. The peak (50%) degree-day requirement to pupation was 218.3 and 193.3 for *O. nubilalis* and *M. cingulum*, respectively. Figure 5.4 suggests a similar synchronization between *M. cingulum* and the multivoltine population at the Landisville location.

O. nubilalis larvae collected near Erie, PA pupated between 75 and 775 degree-days, with 50% pupation at 400 degree-days. Approximately 60% of the Erie population was univoltine and 40% multivoltine. *Ostrinia. nubilalis* larvae collected in Bradford county Pennsylvania began spring pupation at around 75 degree-days and ended pupation at around 750 degree-days, with peak pupation (50%) occurring around 237.5 degree-days. In Bradford county, the *O. nubilalis* population was about 80% multivoltine and 20% univoltine.

5.3.3 *Influence of M. cingulum on O. nubilalis sex ratios*

Across years in Central Pennsylvania and locations within the state in 2003-05, there appears to be a difference in sex ratio between the multivoltine and univoltine portion of the *O. nubilalis* population (Figure 5.5). In general, the presence of *M. cingulum* infestation which was predominantly multivoltine population appeared to have a male bias, while in the absence of *M. cingulum* infestation which was predominantly univoltine population appeared to have a female bias. In Central Pennsylvania, the sex ratio (female/male) in multivoltine population was 0.63, 0.62, and 0.72 in 2003, 2004, and 2005, respectively (Figure 5.6a). The univoltine sex ratio at the same location was 1.72, 1.16, and 1.68 in 2003, 2004, and 2005, respectively (Figure 5.6b). The percentage of the total population parasitized was 15.3, 15.9, and 25.4% in 2003, 2004, and 2005, respectively, in Central Pennsylvania.

At the Lancaster (Landisville, PA), Erie, and Bradford county locations, the sex ratio of the *M. cingulum* infested multivoltine portion of the population was 0.50, 0.60, and 1.10, respectively (Figure 5.7a). In the absence of *M. cingulum* parasitism across degree day groups, the sex ratio was 0.75, 1.75, and 1.30 for the Lancaster, Erie, and

Bradford County collected specimens, respectively (Figure 5.7b). Figure 5.8 illustrates the sex ratio of the temporally separated and spatially isolated *O. nubilalis* populations in the presence or absence of *M. cingulum* infestation.

For 23 out of the 25, 50 degree day interval groups, when *M. cingulum* was present, the sex ratio was male biased (Figure 5.5 and 5.8). In 25 of 40, 50 degree-day interval when *M. cingulum* was absent, the sex ratio was female biased and in 5 additional cases the sex ratio was 1:1. In laboratory studies, the sex ratio was 1:1 for *O. nubilalis*. Thus when most biotic and abiotic factors are controlled, the expected sex ratio is 1:1 for *O. nubilalis.* (Figure 5.9)

Table 5.2 shows comparisons of male and female larval weights, male larval weights compared to the weights of parasitized larvae and female larval weights compared to the weights of parasitized larva for larvae collected in 2005 at Rock Springs, PA in Centre County. Females were significantly heavier than male larvae with males averaging 86.6 mg compared to 111.8 mg for females ($P = 0.001$). Parasitized larvae were significantly heavier than both males and females ($P = 0.001$). When the weights of individual parasitized larvae were plotted against male and female individual larval weights, only about 30% of the parasitized larvae had weights in the range of the unparasitized males, while about 90% of the weights were in the range of the unparasitized female larvae (Figure 5.10). Thus, it appears that *M. cingulum* is differentially parasitizing heavier larvae, which are primarily female.

There was a significant linear relationship between the weight of *O. nubilalis* larvae and the number of *M. cingulum* larvae that emerged from each host (Figure 5.11).

The number of larvae emerging per host ranged from a low of 8 for the lightest *O. nubilalis* larva to approximately 49 for one of the heaviest host larvae.

5.4 Discussions

The results of this study suggest that the *M. cingulum* population in Pennsylvania is adapted to utilize the multivoltine portion of the *O. nubilalis* population as a host. In Central Pennsylvania, the ratio of multivoltine to univoltine individuals in the overall population varies significantly across years. The ratio also varies significantly from one region of the state to another, with each region being dominated by either univoltine or multivoltine populations. The location selected in this study corresponded to locations where *O. nubilalis* were collected by Calvin and Song (1994) to investigate the relationship between location and voltine mix. Their study showed very similar patterns of univoltinism and multivoltinism in the state in 1994.

The selected locations also closely corresponded with another study conducted by Losey et al. (1992) to determine overwintering mortality of *O. nubilalis* in Pennsylvania. They found the highest parasitism rates of *O. nubilalis* by *M. cingulum* in Centre County. In Lancaster County, they observed 14.3% parasitism by *M. cingulum*.

Sked and Calvin (2005) observed an increasing proportion of the population that was univoltine in 2002 at the Center County location. From 1997 to 2001, the percentage of the population that was multivoltine was above 65%, with a high of around 90%. Since 2002, the percentage of the population that is multivoltine has dropped from 60% to about 37.5%. It is unclear what all is shifting the ratio in favor of univoltine individuals, but *M. cingulum* may be involved.

This variation between years and location could be in partly due to *M. cingulum's* synchronization with the multivoltine population, but is likely the result of multiple factors. The climatic at each location determines the average length of a growing season and the amount of variation in growing season length over years. This is turn influences how many generations of a multivoltine population can be completed within a season. Shorter season lengths likely favor the univoltine population, while longer seasons likely favor multivoltine populations. Variation in season length between years may shift the dominance of each voltine type at a given location, but the ratio likely fluctuates around a long term mean ratio. However, climate change could shift the long term equilibrium ratio.

Another factor that may be influencing this change in multivoltine to univoltine ratio is the recent introduction of Bt-corn hybrids. The first Bt-corn hybrids were marketed in 1996. Since that time the percentage of corn acreage planted to these hybrids has increased to about 40% (citation – see earlier chapter). The presence of Bt-corn hybrids could be having a greater impact on multivoltine populations because they are exposed during two or more generations per season compared to one exposure per season for the univoltine population.

With *M. cingulum* differentially parasitizing the multivoltine portion of the population and targeting the females, the combination of *M. cingulum*, the climate, and the introduction of Bt-corn, along with other unknown factors, may be driving the recent trend toward univoltinism in Central Pennsylvania. Clarifying the importance of *M. cingulum* and other factors in voltine pattern shifts will require a more sophisticated analysis then was intended with this study.

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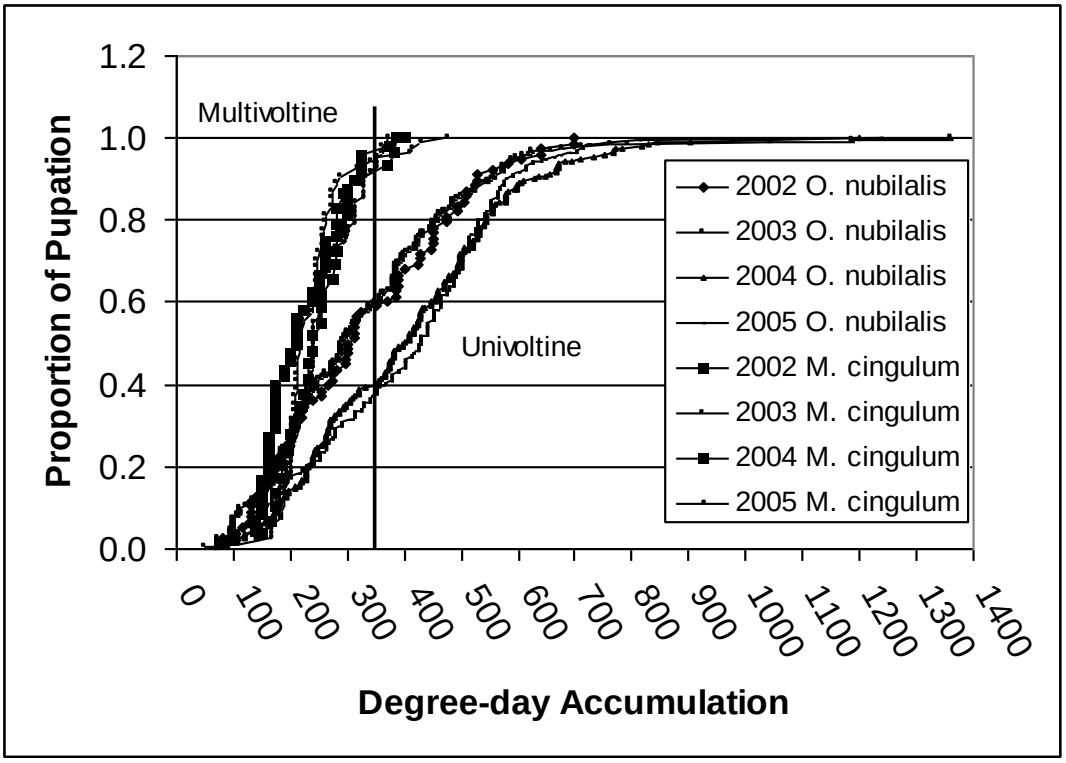
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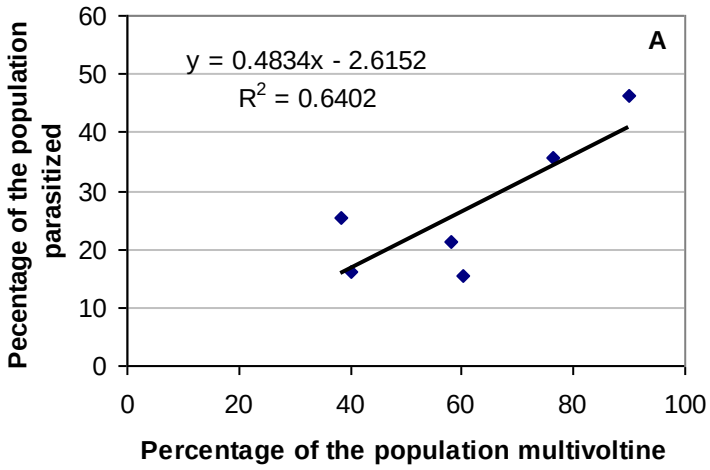
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Fig 5.1: Phenology of the co-occurring univoltine and multivoltine post-diapause *O. nubilalis* and *M. cingulum* spring pupation at 12.5°C base threshold temperature, 18:6 (L:D), and 65% humidity in the environmental chamber, Rock Spring, PA.



* Vertical line on 350 degree day indicates the segregation point of univoltine and multivoltine ecotypes.

Fig 5.2: Relationship between the percentage populations parasitized by *M. cingulum* between 1997-98 and 2002-05. (A) percentage of ECB population that was multivoltine verses the percentage of the total population parasitized (B) percentage of corrected multivoltine (ECB + parasitized larvae) population verses the percentage of the multivoltine population parasitized



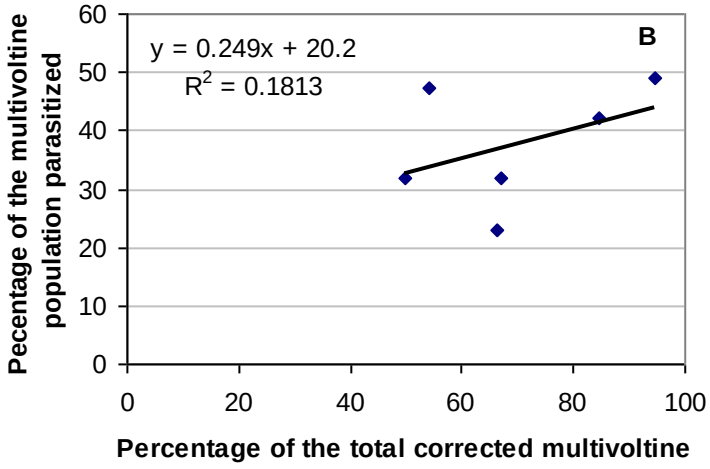
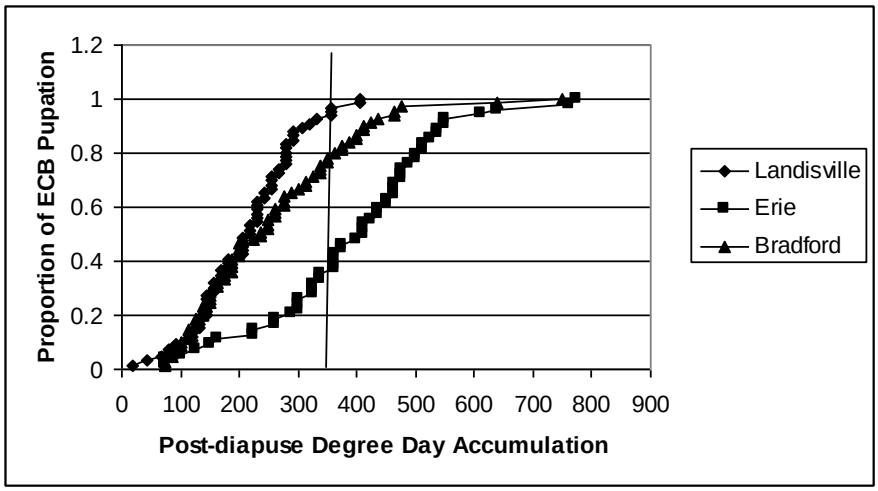


Fig. 5.3: Phenology of the spatially isolated multivoltine and univoltine ecotypes post-diapause *O. nubilalis* spring pupation at 12.5°C base threshold temperature, 18:6 (L:D), and 65% humidity in the environmental chamber.



* Vertical line on 350 degree day indicates the segregation point of univoltine and multivoltine ecotypes.

Fig. 5.4: Phenology of the post-diapause multivoltine *O. nubilalis* and *M. cingulum* spring pupation at 12.5°C base threshold temperature, 18:6 (L:D), and 65% humidity in the environmental chamber collected from Landisville, PA.

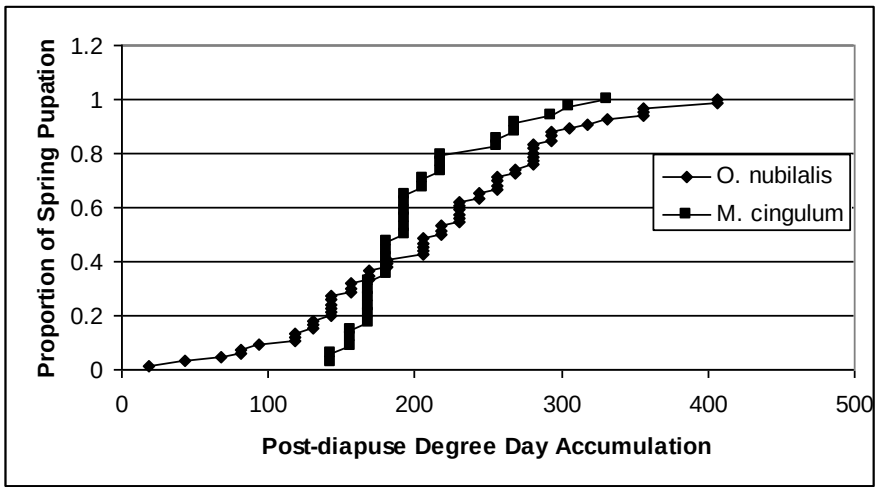
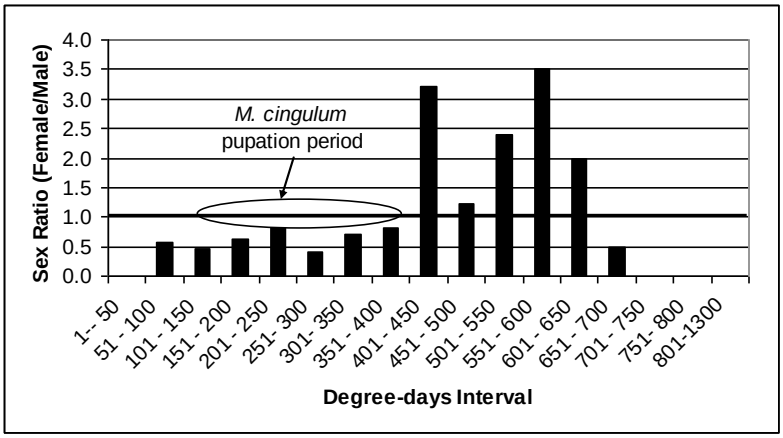
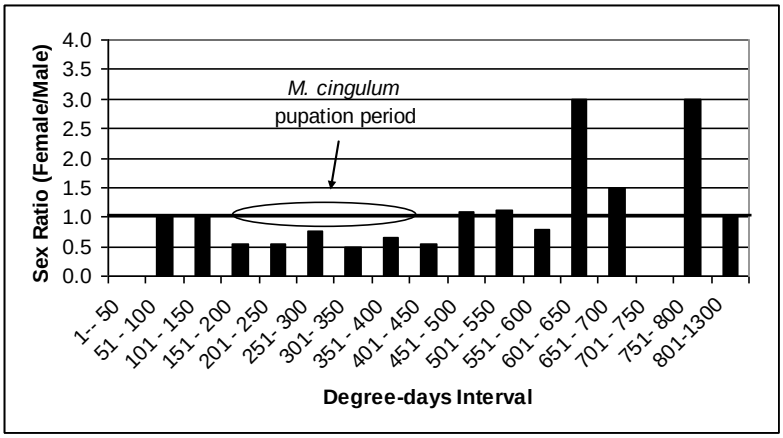
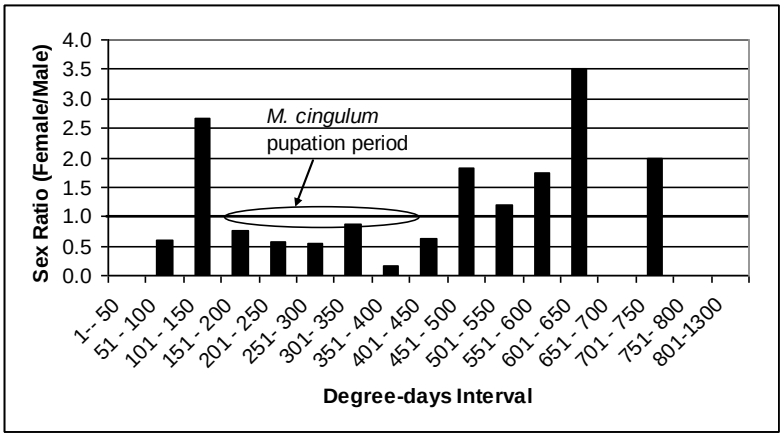


Fig. 5.5: Sex ratio changes in post-diapause temporally separated *O. nubilalis* ecotype population over time (50 DD interval groups) in the infestation periods of *M. cingulum* parasitoid. (A) 2003 (B) 2004 and (C) 2005 in Rock spring, PA

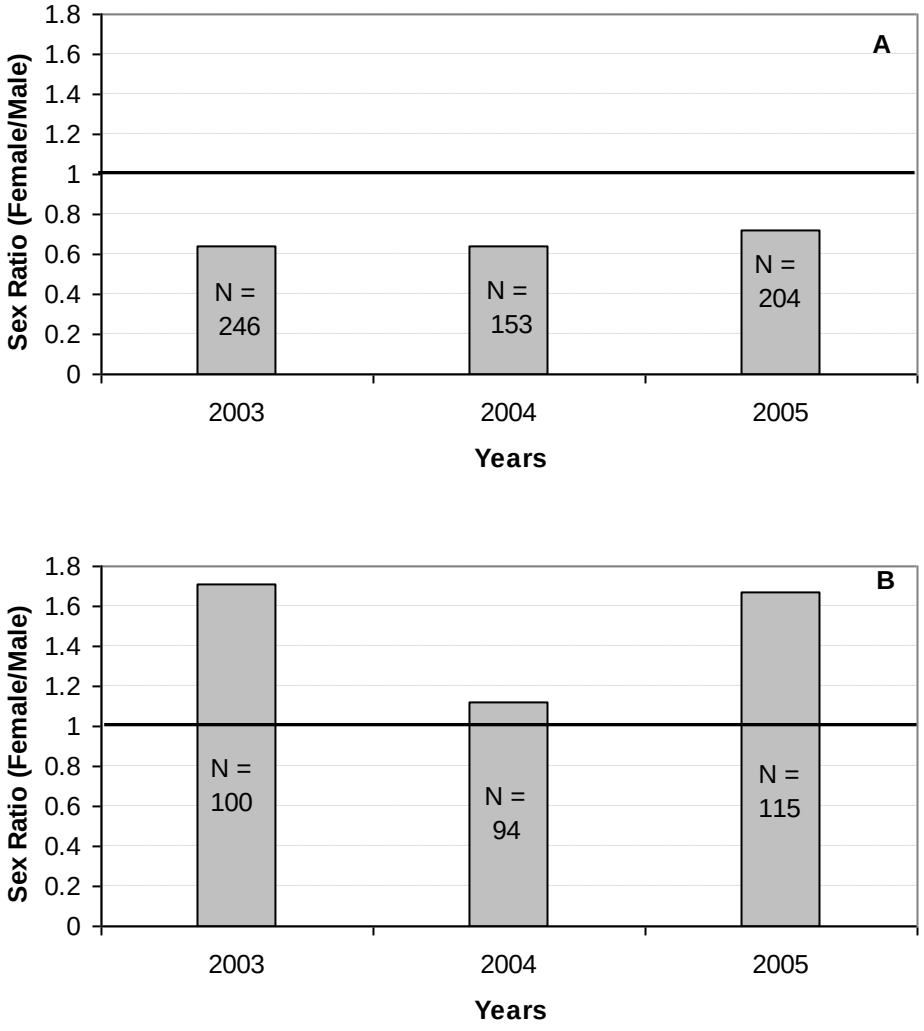






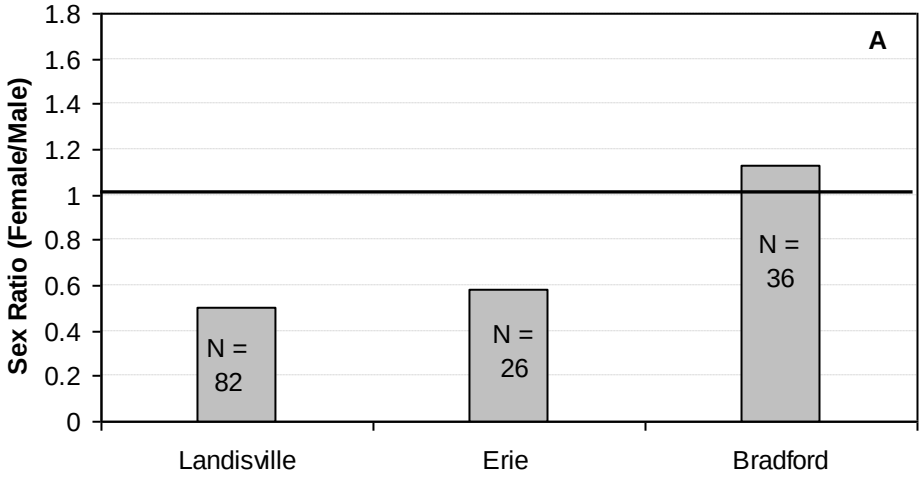
* Horizontal lines on sex ratio scale at 1.0 position shows equal sex ratio.

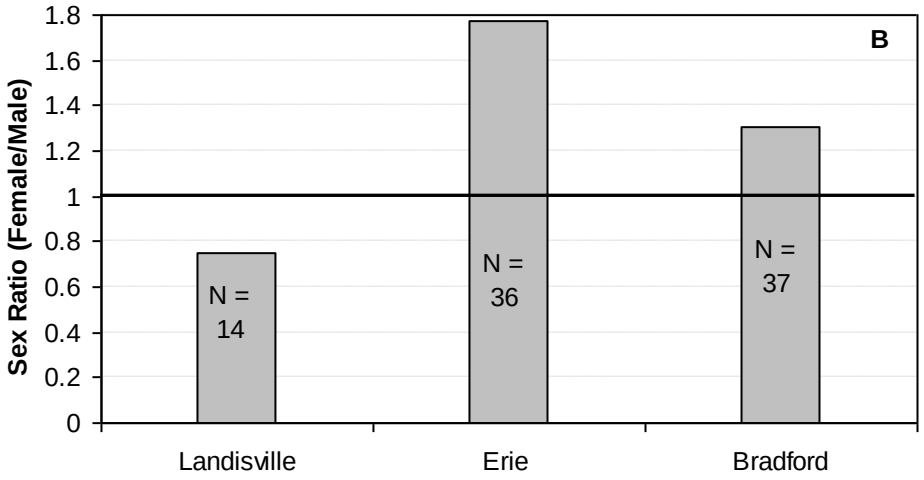
Fig. 5.6: Sex ratio of *adult O. nubilalis* emerged during period of (A) presence verse (B) absence of the parasitoid *Macrocentrus cingulum* in the Central region of Pennsylvania



* Horizontal lines on sex ratio scale at 1.0 position indicates equal sex ratio

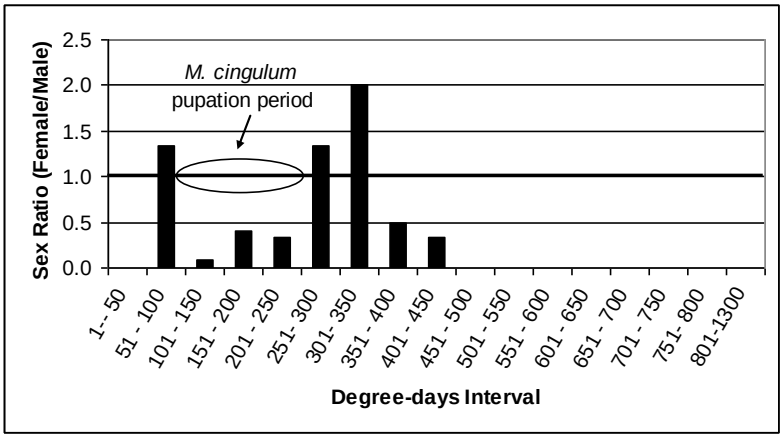
Fig 5.7: Sex ratio of *adult O. nubilalis* emerged during the period of (A) presence verses (B) absence of the parasitoid *M. cingulum* in three spatially isolated ecotype regions of Pennsylvania

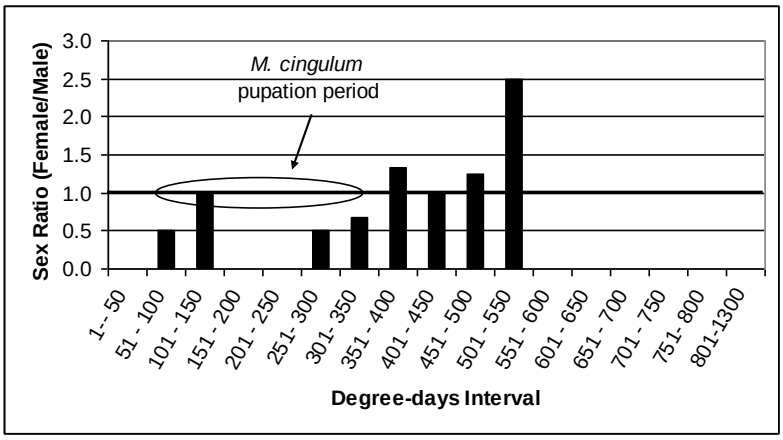


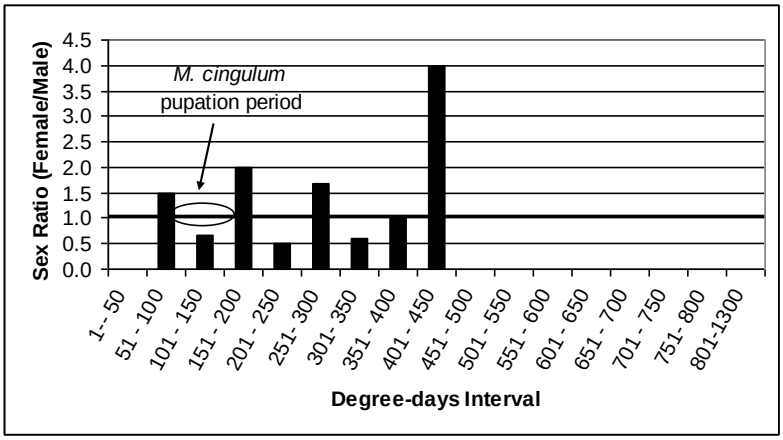


* Horizontal lines on sex ratio scale at 1.0 position indicates equal sex ratio

Fig. 5.8: Sex ratio changes in temporally separated but spatially isolated post-diapause *O. nubilalis* ecotype populations over time (50 DD interval groups) in the infestation period of *M. cingulum* parasitoid in 2005. (A) Landisville, PA (B) Erie, PA (C) Bradford, PA.







* Horizontal lines on sex ratio scale at 1.0 position indicate equal sex ratio.

Fig 5.9: Sex ratio of adult ECB emerged in the non-diapause (F_1) condition reared in environmental chamber in Laboratory

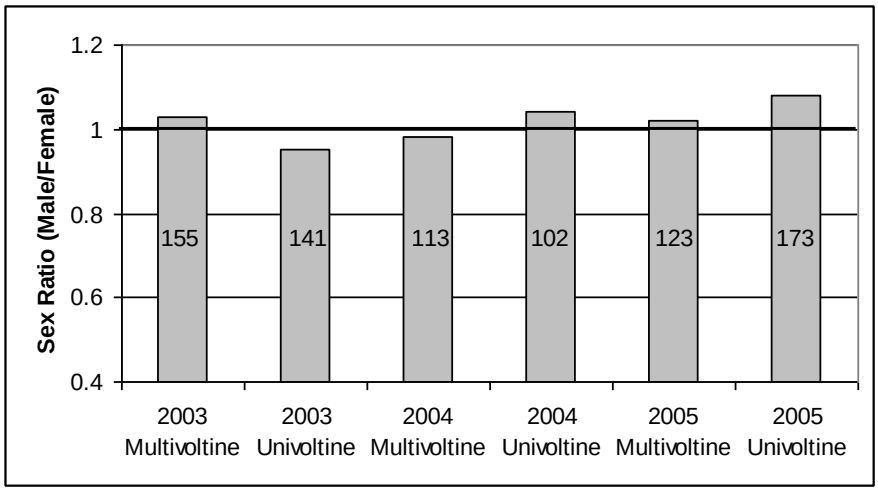


Fig 5.10: Post-diapause larval weight comparison between male, female and parasitized ECB larva in 2005

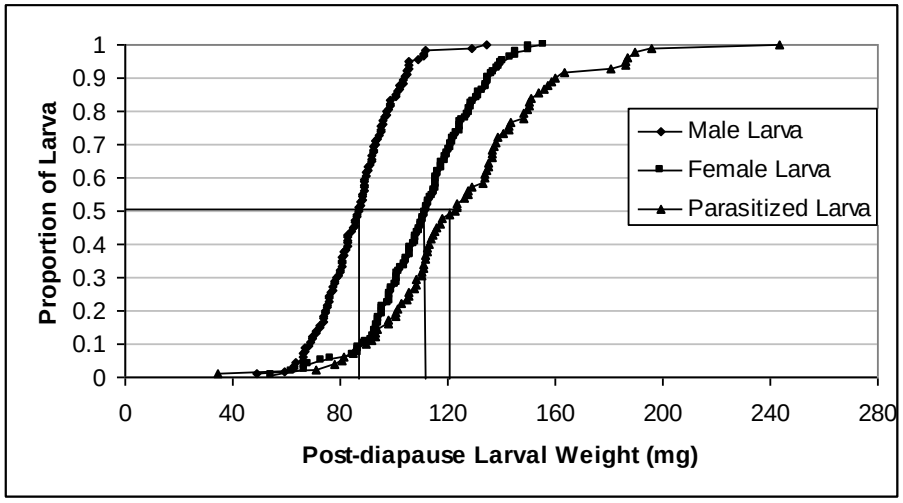


Fig 5.11: Relationship between parasitoid, *M. cingulum* infested post-diapause *O. nubilalis* larval weight and the number of parasitoid emerged at Rock spring, PA 2005

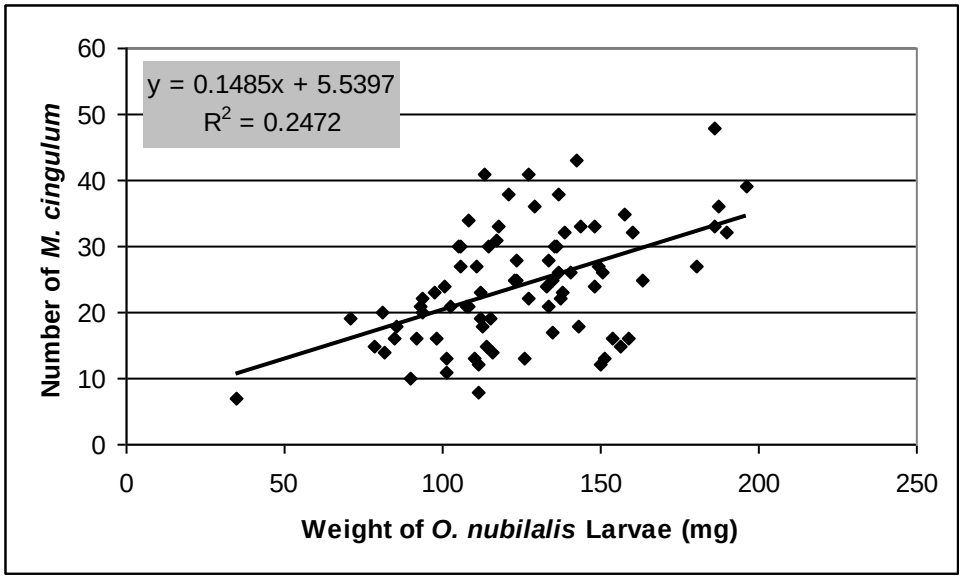


Table 5.1: Number of collected *O. nubilalis* or *M. cingulum* larva pupated and the proportion of multivoltine and univoltine ecotypes in the sympatric region of central Pennsylvania

Year (s)/Locations	Number of collected larvae survived	Number of *O. nubilalis* pupated	Number of larva parasitized by *M. cingulum*	% of *M. cingulum* Infestation	Proportion of Multivoltine	Proportion of Univoltine
1997 Rock spring	160	103	57	35.62	76.25	23.75
1998 Rock spring	268	144	124	46.26	90.00	10.00
2002 Rock spring	127	100	27	27.10	58.00	42.00
2003 Rock spring	406	344	62	15.3	60.22	39.78
2004 Rock spring	301	253	48	15.94	40.31	59.69
2005 Rock spring	322	240	82	25.46	38.33	61.67
2005 Landisville	103	66	34	34.0	97.0	3.0
2005 Erie	61	54	7	11.5	33.2	66.8
2005 Bradford	72	68	4	5.5	76.8	23.2

Table 5.2: Post-diapause weight comparison between male, female and parasitized larva collected from the fields of Rock Spring, PA in 2005

Larval weight comparison	Number of larvae (N)	Mean (SE)	P value
Male larva verses Female larva	114 123	86.6 (1.3) 111.8 (1.7)	0.001*
Male larva verses Parasitized larva	114 82	86.6 (1.3) 126.0 (3.5)	0.001*
Female larva verses Parasitized larva	123 82	111.8 (1.7) 126.0 (3.5)	0.001*

Chapter 6.

Transition between univoltine and multivoltine European corn borer (*Ostrinia nubilalis* Hübner) ecotype populations: the role of seasonal degree-days and genetics.

6.1 Introduction.

Understanding the complex interaction between genetic traits and environmentally induced variation in traits is the key to understand the evolutionary and ecological significance of various life history characters including diapause in insect species. Post-diapause development period for univoltine European corn borer (ECB) was longer then post-diapause developmental period of bivoltine ECB under the same environmental conditions (Thomas et al. 1992, Zaman Ph.D dissertation chapter 2). Diapause induction and termination in European corn borer (ECB), *Ostrinia nubilalis* Hübner, ecotypes is believed to be controlled by the combination of genetic traits and environmental factors (Beck and Hanec 1960, McLeod et al. 1978, Reed et al. 1981). Lack of information of those complex interactions is partly responsible for the uncertainty in predicting the phenotype and behavior of most organisms (Scriber 1994, Tauber et al. 1986). Knowledge about the influence of genetic traits in response to the environmental components such as seasonal degree-day accumulations on the diapause traits of different ecotypes of *O. nubilalis* species will further explain the interactions among various life history traits of this organism.

First noticed in 1917 near Boston, European corn borer was accidentally introduced in North America by the importation of broom corn from Hungary and Italy (Smith 1920, Baker et al. 1949). During the time of its introduction (1909-1914), the European corn borer produced only one generation in each year (Fitch 1936, Vance

1942). With in a decade during late 1930s a two generation per year ECB population started to appear in the eastern and north states of the U.S. At present, a univoltine or one generation per year population appeared to be common in north of U.S. while a multivoltine or three to four generations per year appeared to be common in the southern states (Showers et al. 1975, Mason et al. 1996)

European corn borer voltine ecotypes developed in responses to the photoperiod and temperature with respect to diapause induction, termination and the degree-days to pupation (Showers 1981). In the cooler regions, fifth instar ECB larva goes into diapause triggered by the photoperiod, temperature, genetic composition of the population and perhaps by the nutritional status of the host plant. In most phytophagous insects, besides the environmental components host plant preference showed significant effects on voltinism pattern. For example, insects that feed on roots or tree trunks often required longer period even several months for development then those feeding on soft tissues such as leaves (Haack and Slansky, 1987). Larval growth was found to be slower in species that feeds on mature leaves (leaves with decreased nitrogen and less succulent) than the younger leaves (Scriber and Slansky 1981, Scriber and Feeny 1979). The number of generation possible in a geographic location depends therefore, on the host plant status as well as the length of growing season (Scriber and Hainze 1987, Scriber and Lederhouse 1992).

Ostrinia nubilalis adults are not long distance flyers, and latitudinal barriers (such as mountain ranges) restrict inter-specific voltine ecotype migration among regions of north to south of U.S. and vice versa (Chiang 1965, McEwen et al. 1968, Chiang 1972, Showers 1979, 1993). At present, cooler temperatures and shorter growing seasons in the

northern U.S. univoltine European corn borer populations have an advantage, though there is a risk of higher mortality from extended larval dormancy. However in central states and in southern warmer states multivoltine European corn borer populations are dominant. Variation in post diapause development and adult emergence periods between voltine populations (Calvin and Song 1994, Hoard and Weiss 1995) and restricted migration due to latitudinal barriers might create asynchrony in mating between voltine populations, and that might limit gene exchange between voltine populations (Roelofs et al. 1985).

However, future climate change may influence *O. nubilalis* numbers of generations per year, voltine pattern, timing as well as length of insect activities of a particular region. Climate change may shift the present feeding status of the pest. Ecotype population feeding on different host plants or feeds on different stage of a host may face reproductive isolation due to different emergence period. Adaptation to various environments could lead to rapid speciation of *O. nubilalis*. Studies suggested that sympatric speciation might occur through differential adaptations to various environments (Bush 1994, Orr and Smith 1998, Schluter 1998, Via 2001, Berlocher and Feder 2002) and that speciation could happen rapidly through ecological selection (Via 2002). Recent example of rapid genetic divergence, reproductive isolation, and potential ecological speciation involving host plant specialization was observed in *Rhagoletis spp* (Filchak et al. 2000, Berlocher and Feder 2002, Schwarz et al. 2003) and in pea aphids (Caillaud and Via 2000, Via 2001, 2002). Recent specialization in sex pheromone preference in sympatric population was observed in European corn borer (Roelofs et al.

1985, Roelofs et al. 2002, Thomas et al. 2003, Bethenod et al. 2005) and very recently in Larch bud moths (Emelianov et al. 2001).

Climate change is not a speculation anymore rather a reality. Numerous studies suggested an increase in global temperature in future. Prior understanding of the *O. nubilalis* population response in environmental change will help to improvise the current pest management system. Our study was an attempt to explore the role of seasonal degree-day as the regulatory components of European corn borer voltine pattern in a temporally isolated population. In this study we assessed changes in the patterns of univoltine and multivoltine European corn borer ecotypes with respect to seasonal degree-day accumulation in central Pennsylvania for the last ten years (1996- 2005). We also evaluated whether differences in degree day accumulation among temporally isolated voltine ecotypes could be explained by variation observed within and among families from univoltine and multivoltine individuals.

6.2 Materials and Methods:

6.2.1 Colony Collection and Rearing:

The European corn borers, *Ostrinia nubilalis*, were collected as over wintering larvae from corn stubble in several fields at the Russell E. Larsen research center at Rock Spring, Pennsylvania. Rock Spring is located at latitude 40.82^{o} N, and longitude 77.94^{o} W at an elevation of 1212 feet in the valley of Appalachian mountains, 12 miles south of the Penn State University main campus. Between mid-Aprils to early-May, 1997-1998 and 2002-05, each year 127- 450 diapausing 5th-instars larvae were collected from corn stubble. Field collected larvae were individually placed in a 30 ml plastic cup, and then

transferred to the Department of Entomology at the Pennsylvania State University. A water soaked small cotton ball was placed in each cup to provide sufficient moisture and prevent desiccation and entrance to diapause (Babcock 1924, 1927, Mellanby 1958, Beck 1967). After preparation cups were kept in an environmental growth chamber (Revco model no.RI-23-555-A) at 25^0C ($\pm 0.5^0$C) and 18L: 6D photoperiod. No diet was provided during the post-diapause development period. Larvae in each cup were marked with a serial number and the date of collection was recoded to calculate the exact number of degree-day that a larva experienced while in the growth chamber. To avoid contamination aseptic condition conditions were maintained for the entire procedure from field collection to adult oviposition.

Larvae were examined daily for pupation, parasitoid infestation, and death caused by disease or injury during the collection process. The fates of field collected larvae were explained using the categories described by Losey et al (1992). Larvae exposed to any unusual circumstances such as disease, injury, or parasitoids were isolated to avoid contamination. When an individual European corn borer pupa found, the date of pupation was recorded. A pupa was left in the growth chamber until it emerged as an adult and the date of emergence was recorded.

When collected in the field, it was not possible to determine if a larva is univoltine or multivoltine. Univoltine and multivoltine ecotypes were separated by the methods described in Chapter 2 followed by the method developed by Calvin and Song (1994). Univoltine *O. nubilalis* required between ≥351 degree-days where as multivoltine *O. nubilalis* required ≤ 350 degree days. Degree-days were calculated using the methods described by Arnold (1959). A base threshold temperature of 12.5^0C was

used for *O. nubilalis* development (Calvin *et. al*. 1991). The total degree day calculation and mathematical model construction were described in section 2.2.3 in chapter 2.

6.2.2 Family studies:

In 2003, about two hundred and ninety four adults emerged from the field-collected larva and adults were segregated into multivoltine and univoltine groups. Adults resulting from 50 degree days to 600 degree-days were separated into 11 groups based on 50 degree day intervals. Adults resulting from ≤350 degree-days pupation interval groups were designated as multivoltine and adults resulting from 351 – 600 degree-days pupation interval groups were designated as univoltine. From these adults, paired mating were established. Random pairings among adults were not possible due to the nature of the degree-day accumulation. The degree-day accumulations of parents were therefore highly correlated. Field collected, over-wintered larvae started pupating at around 75 degree-days. Although, some portion of the collected larvae (7%) pupated above 600 degree-days to as high as 1400 degree-days, establishing viable mating pairs was not possible for the low numbers of adults emerging within this interval. Resulting pupae were left in the growth chamber until adult emergence.

Each pair of adults were transferred into individual cylindrical cages (3.5 inches in height and 3.0 inches in diameter) made with 286-mesh size aluminum screen. The upper sides of the mating cages were covered with 64-mesh screen that facilitated females' ovipositing onto wax paper. The cages with the adults were placed into a walk-in environmental chamber (Revco model no. RI-23-555-A) maintained at 25^0C ($\pm$ 0.5^0C) and 18L: 6D photoperiod and >90% relative humidity. Relative humidity was maintained

using a volume-controlled humidifier (Vicks vaporizer, model 150) inside the environmental chamber. Periodic humidity measurement was done using a digital humidity meter (Labcraft brand, model 264-767) to assure high enough levels for oviposition. The cages were also misted daily to ensure adequate moisture, and the adults were provided honey solution to maximize fecundity and longevity (Leahy and Andow 1994). Wax paper (Reynolds Cut-Rite brand) was placed on the top of each mating case for oviposition and collection of egg masses. Eggs deposited on wax paper were collected at two to three days intervals depending upon the number of egg masses laid. Egg masses from each field collected parent family were placed in a screen-topped Mason jar with a moist towel inside and incubated until larvae hatched.

Viable offspring were available from F_1 families of *Ostrinia nubilalis*. Fifteen randomly selected neonatal larvae from each family were reared individually in 30 ml plastic cup with 6gms of artificial diet inside. A total of 750 neonate larvae from 50 families were reared in the same environmental condition as their parents. Degree-days for pupation of those first generation larvae were calculated following the method described earlier in chapter 2.

6.2.3. Data Analysis:

Regression analysis were done between years and proportion of multivoltine and univoltine populations pupated during 1997 – 2005. A regressions analysis was done between the standard deviation of the cumulative degree-days required for offspring egg hatch to pupation and mid-parents pupation degree-days (Figure 6.4).

Familial patterns for degree-day accumulation of offspring were examined using analysis of variance (ANOVA). The purpose of the ANOVA was to test several hypotheses: (1) Do offspring from different families differ for degree-day phenotype? ("Family" effects); (2) Are degree-day phenotypes different for the offspring of univoltine and multivoltine classifications of parents? ("Voltine differences"); (3) Are univoltine families different in their degree-day phenotypes? ("Differences among univoltine families"); and (4) Are multivoltine families different in their degree-day phenotypes? ("Differences among multivoltine families"). Degree days were $\log_{10}$-transformed to enhance normality. However, even after transformation, families did not exhibit common variances for degree days. To account for the heterogeneity of variances among families, ANOVA using restricted-likelihood estimates were performed using Proc Mixed of the statitistical package SAS, v. 9.1. Proc Mixed allows variance heterogeneity to be incorporated into the model by specifying different variances or covariance structures for different levels of a factor (Littell et al. 1998). Family effects (49 dfs) were considered fixed and the "Repeated" statement of Proc Mixed was used to estimate separate variances for each family ("group" option where group= family). Linear-contrast statements were used to partition family differences (49 dfs) into three independent, orthogonal comparisons: (1). Voltine Differences (1df); (2). Differences among univoltine families (20 dfs); and (3). "differences among multivoltine families" (28 dfs).

6.3 Results:

6.3.1 Post-diapause development:

Seven years post diapause development patterns showed that over wintered larvae started pupation between 50 to 75 degree-day accumulations but the degree-days requirement for 50% pupation varied significantly between years (1997-2005) (Figure 6.1). Total degree-day accumulations also varied between years, as low as 887.5^{o}C degree-days were observed in 1997, and as high as 1200^{o}C DD were observed in 2002. A trend of increasing degree-day accumulations was observed in the Rock Spring area within the study period (1996-2005), with an average of 1009.6^{o}C degree-days between 1996 to 2000 to 1086.36^{o}C degree-days between 2001 to 2005 (Table 6.1). Proportion of ecotype also changed over the study period. Degree-day requirement for 50% pupation were increased (Table 6.2) during the study period. As the trend increased between 1996 and 2005, the 50% pupation period also increased from 215.28^{o}C DD in 1997 to 425.5^{o}C DD in 2005. Although, the degree-day requirements for the initial population (first 5% time to pupation) among years did not vary significantly ($p = 0.142$), the degree-day requirements for the later portion of population (last 50% -100% time to pupation) varied significantly ($p = <0.05$) among the years (Table 6.2). Between 1997-2005 proportion of univotine ecotype in the overall ECB population at central PA was increased significantly ($R^2 = 0.664$; Fig. 6.2b). On the other hand proportion of multivoltine ecotypes decreased ($R^2 = 0.664$; Fig. 6.2a).

6.3.3 Family analysis:

Families differed in their degree days necessary for pupation ($F = 17.26$. $df = 48, 161$, $P<0.0001$). Offspring of univoltine parents required significantly more degree days

than the offspring of multivoltine parents ($F = 63.67$, df =1, 104, $P<0.0001$; Fig. 6.2). However, there were also significant differences among families within the univoltine groups of adults ($F = 3.00$, df = 20, 67.7, $P = 0.0005$) and the multivoltine groups ($F = 16.28$, df = 28, 104, $P <0.000$; Fig. 6.3). Regressions analysis between the standard deviation of the cumulative degree-days of offspring's pupation and the degree-days of mid-parents pupation showed that offspring's from the lower degree days required individual had the lower standard deviation than that of the offspring's from the higher degree day required parents (Figure 6.4).

6.4 Discussions:

Several trends were observed over the last nine years where seasonal degree day and post diapause *O. nubilalis* post diapause data were available for the study site in central Pennsylvania. As the degree-day accumulation increased over the last ten years (1996-2005), the degree-day requirements for peak (50%) European corn borer pupation was increased. In 1997, fifty percent of the overwintering European corn borer larvae pupated at 215.8 degree-day, whereas in 2005 fifty percent of overwintering larvae pupated at 425.50 degree-days. As the growing season progressed from January 1 of the year to the onward more heat units or degree-days have accumulated thus the number high degree day required individuals (essentially the univoltine portion) might get higher opportunity to increased its proportion in the over all ECB population, thus proportion of lower degree day required individuals (essentially the multivoltine portion) might get decreased in the over all population. Several phenomenons could explain the situation; first, as the degree day accumulation in a region increases, diapausing European corn borer population might select in favor of higher degree day accumulation. Secondly,

higher degree day accumulation in a region might lower the risk of dormant (overwintered) larval mortality thus increasing the high degree-day requiring individuals in the ECB population of the region. A third possibility is that higher parasitoid infestations in the multivoltine or early portion of the general population may select against the lower degree-day requiring individuals thus reducing the lower degree-day proportion in the population.

Field collected over wintered larval pupation pattern showed that increased degree-day accumulations in a geographical area also prolonged the pupation period of the overall population of that geographical area (figure 6.1) might favoring genes that prolong the post diapause larval period. As an example, we observed that the last 10% of the population to pupate required at least 478.61, 362.50, and 412.39 degree days in 1997, 1998, and in 2001, respectively. However, in 2003, 2004, and 2005 the last 10% of the population required at least 558.50, 603.72, and 576.66^{o}C degree-days, respectively (table 1). Years with higher degree-day accumulations (warmer year) would allow larvae to pupate that would normally be killed at the end of the growing season.

Typically the tail of the univoltine population curve (fig 2a) is extended due to a higher proportion of larvae that required more degree-days to pupation. Because, 1997 was a cooler season (only 887.5 DD have accumulated), it did not favored the high degree day individual thus ECB population in central PA might received lower univoltine genetic component transfer to the next year and we observed an increase in the lower degree-day requiring or multivoltine component in the population (88.88%) and a decrease in higher degree-day requiring or univoltine component in 1998 ECB population (table 1).

From the results observed in this study, it is clear that variation in post diapause developmental time is a heritable character in the *O. nubilalis* species. Future experiments with reciprocal crossing between univoltine and bivoltine parents and subsequent paternal and maternal backcrossing with multiple generations may further explore the genetic basis and sex linked inheritance of the voltine populations of European corn borer.

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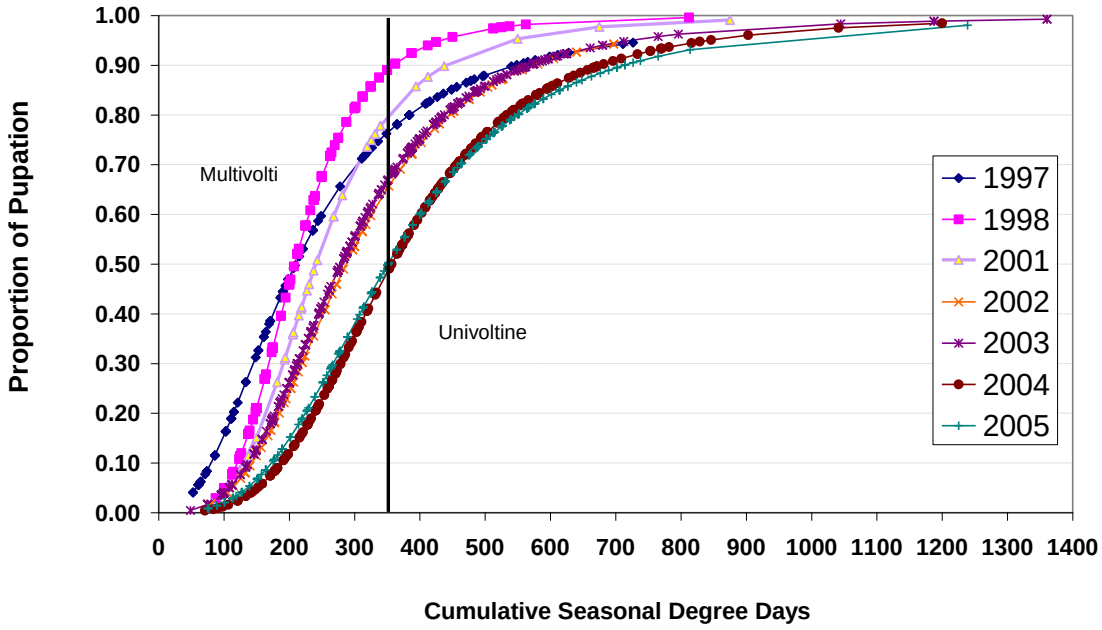
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Figure 6.1: Seven Years of observed European corn borer pupation patterns at Rock spring, PA



* Vertical line on 350 degree days is the segregation point between multivoltine and univoltine ecotypes

Figure: 6.2 Proportional changes of (A) multivoltine and (B) univoltine ecotypes over the last 9 years at Rock Spring in central Pennsylvania.

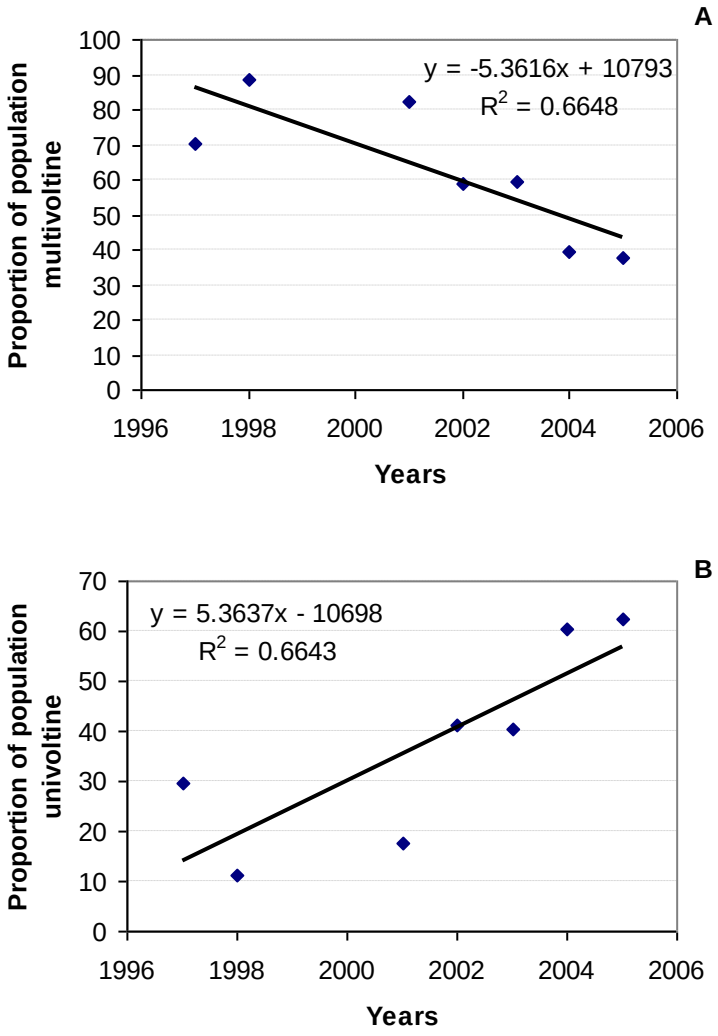
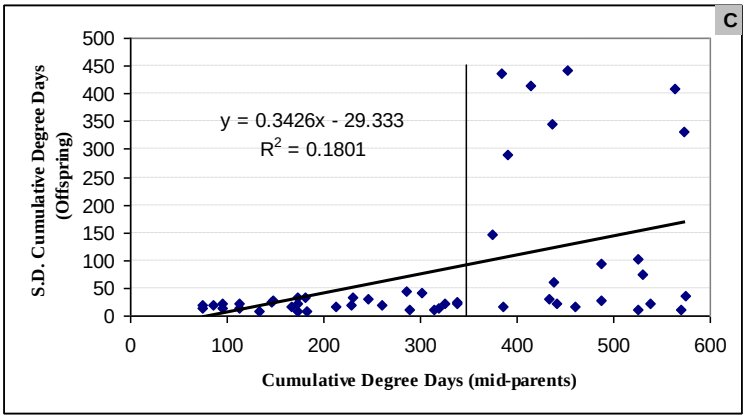


Figure 6.3 Regressions between the standard deviation of the cumulative degree-days required for offspring egg hatch to pupation and mid-parents pupation degree-days. Fifteen offspring were measured in each of the 50 families



* Vertical line on 350 degree day position is the segregation point between multivoltine and univoltine ecotypes

Figure 6.4 Box plot showing the family estimates of offspring's degree day (log data) in relations to their voltine parents.

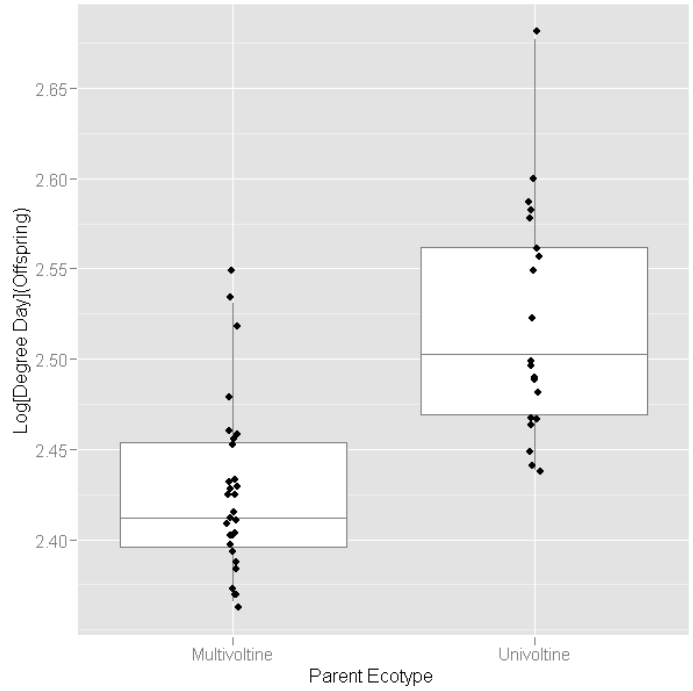


Figure 6.5 Box plot showing the variance in family estimates of offspring's degree day in relations to their voltine parents.

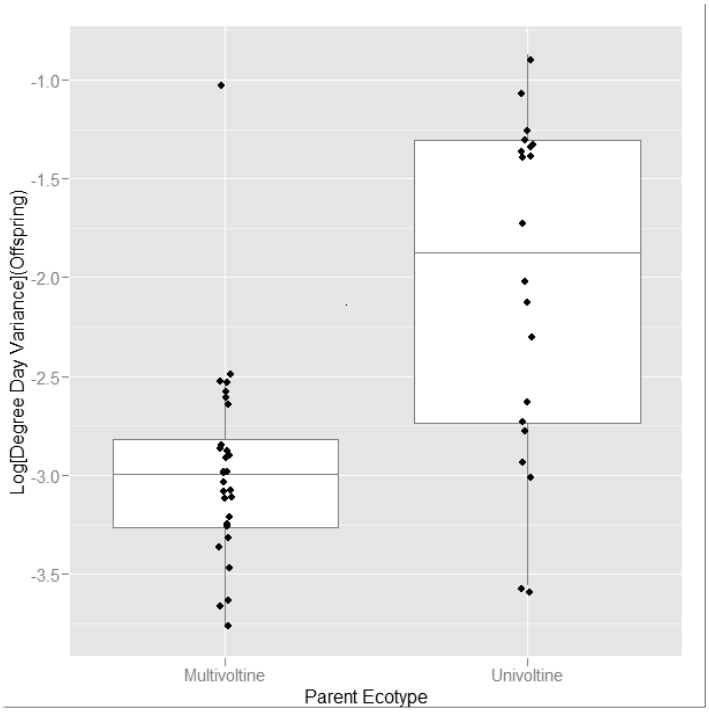


Table 6.1 Degree-day accumulation, proportion of observed pupation, and proportion of bivoltine and univoltine European corn borer populations at rock spring, PA.

Year	Degree day Accumulation	5% Pupation	50% Pupation	90% Pupation	95% Pupation	Proportion of Bivoltine (%)	Proportion of Univoltine (%)
2005	1189.2	118.77	425.50	576.66	648.27	37.50	62.50
2004	1067.2	141.22	403.06	603.72	690.00	39.50	60.50
2003	953.6	100.00	288.72	558.50	597.11	59.64	40.36
2002	1200.6	114.94	296.00	527.60	604.94	58.60	41.40
2001	1021.4	137.39	227.10	412.39	550.00	82.35	17.65
1998	1127.8	112.5	207.28	362.50	449.49	88.88	11.12
1997	887.5	61.11	215.28	478.61	576.67	70.20	29.80
Mean	1063.9	112.27	294.70	502.85	588.06	62.36	37.61
SEM	44.60	10.10	33.59	33.73	28.93	7.43	7.44
P-value		P=0.14	P=0.008	P=0.03	P=0.04	P=0.02	P=0 .02

Chapter 7.

Conclusions and future research.

This study clearly demonstrates that some aspects of the life history parameters of co-occurring univoltine and multivoltine European corn borer ecotypes are different. Key life history differences between univoltine and multivoltine ecotypes are the length of developmental period, larval and pupal growth and synchronization with parasitoid (*M. cingulum*) emergence. The preceding chapters have described the influence of seasonal degree days, parasitoid infestation, and *Bt* toxin susceptibility on the life history of European corn borer ecotypes. Some questions remain unanswered in this dissertation and need more sophisticated research and conceptual model construction. In the following section I will summarize major findings of this dissertation research and focus on some future research to address some of the unanswered questions.

7.1 *Variation in post-diapause and non-diapause development*

Data presented in chapter 2 clearly indicate that across the years over-wintering univoltine and multivoltine ecotypes started pupation at the same period at 75.0 DD and ended at as high as 1360.0 DD accumulation. When multiple generations of segregated ecotype populations were reared on artificial diet and in controlled conditions, univoltine population required a wide range of degree-days to complete pupation than multivoltine (chapter 2). When progeny of monogamously mated pairs of field collected post-diapause univoltine and multivoltine population were reared in controlled non-diapause environments they showed a clear relationship with their parent's degree-day requirements (chapter 6). Univoltine offspring required more degree-days to enter

pupation than the multivoltine offspring. Variation in non-diapause development patterns between univoltine and multivoltine ecotypes suggested the presence of a genetic basis for higher degree-day requirements for the univoltine component in the overall European corn borer population. We also observed an increase in the univoltine portion in the overall ECB population in central PA during 1997-2005. Increase in univoltine portion might be influenced by the higher degree day accumulation in central PA region over the last 10 years (chapter 6). So, the post-diapause individual requiring more degree-days to pupate may contribute more of the univoltine genotype to the over all ECB population thus increasing the proportion of univoltine ecotypes. Further reciprocal crossing between ecotypes and following the progeny's degree-day requirement patterns for several generations may confirm the genetic basis of this life history character.

7.2 Growth and reproductive parameters comparison

The study found that post-diapause univoltine larvae and pupae are significantly heavier than those of multivoltine. This means that despite long-term diapause, univoltine individuals do not compromise their larval and pupal weight. Although, higher larval and pupal weight was observed in individuals of univoltine ecotypes, reproductive parameters such as oviposition period, longevity and egg deposition was not significantly different between ecotypes. This experiment was done in laboratory conditions and that might limit the reproductive potential of adult ECB. It is possible that longevity and oviposition may be influenced by larval feeding on different host plant stages and adult food sources because of variable emergence periods during the corn growing season. When non-diapausing univoltine and multivoltine ecotypes were reared on artificial diet in a

controlled environment no significant pupal weights variation was observed. The results suggested a non-genetic control of larval and pupal weight differences between ecotypes meaning that environment is a significant factor. Further experiments feeding tissues from different stages of corn plants may elucidate the growth and developmental variation between ecotypes. A future reproductive parameter evaluation under field conditions with natural food may reveal the consequences of those variable larval and pupal weights.

7.3 Response to Cry1Ab and Cry1F Bt toxin

No significant susceptibility differences were observed between neonates of univoltine and multivoltine ecotypes subjected to Cry1Ab and Cry1F *Bt* toxin. A small increase in Cry1Ab susceptibility (1.5-2.0 folds) in univoltine ecotype is not an indication of resistance development, rather it is considered natural variation in the ECB population. When neonates were exposed to the lower concentrations (less than LC_{50}) of Cry1Ab and Cry1F Bt toxins, sublethal effects were not significantly difference between the ecotypes. The consequences of these sublethal effects on the fitness of ECB life history i.e. development period, life cycle completion, oviposition rate etc. were not evaluated in this experiment. Future evaluation with multi ecotype populations across several geographic areas and multi generational studies are needed to strengthen the current Integrated Resistance Management (IRM) strategies.

7.4 Impact of M. cingulum parasitization

The results of this study suggest that the *M. cingulum* population in Pennsylvania is adapted to utilize the multivoltine portion of the European corn borer population as a host. The ratio of univoltine and multivoltine varies significantly across years and from one region of the state to another, with each year and region being dominated by either univoltine or multivoltine populations. This variation between years and locations could be in partly due to *M. cingulum's* synchronization with the multivoltine population, but is likely the result of multiple factors. Sex ratio differences observed in over-wintered ECB populations in the presence or absence of *M. cingulum* parasitism suggested differential parasitism between males and females. A choice test in the laboratory between univoltine and multivoltine will be the next step to confirm *M. cingulum* preference.

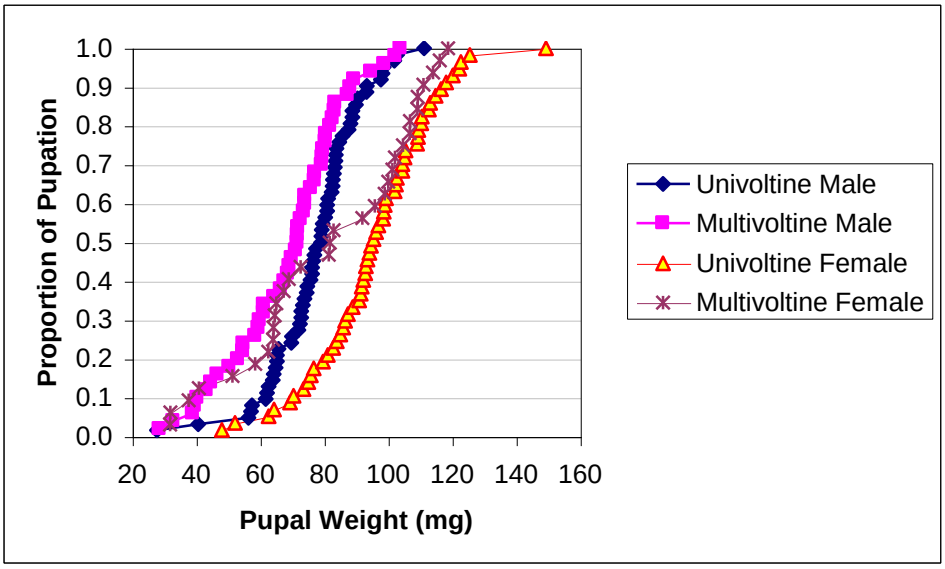
7.5 Transition between voltine populations in region.

With *M. cingulum* differentially parasitizing the multivoltine portion of the population and targeting the females, the combination of *M. cingulum*, climate, and the introduction of Bt-corn, along with other unknown factors, may be driving the recent trend toward univoltinism in Central Pennsylvania. Clarifying the importance of *M. cingulum* and other factors in the voltine pattern shifts will require a long term data collection more sophisticated analysis then was intended with this study.

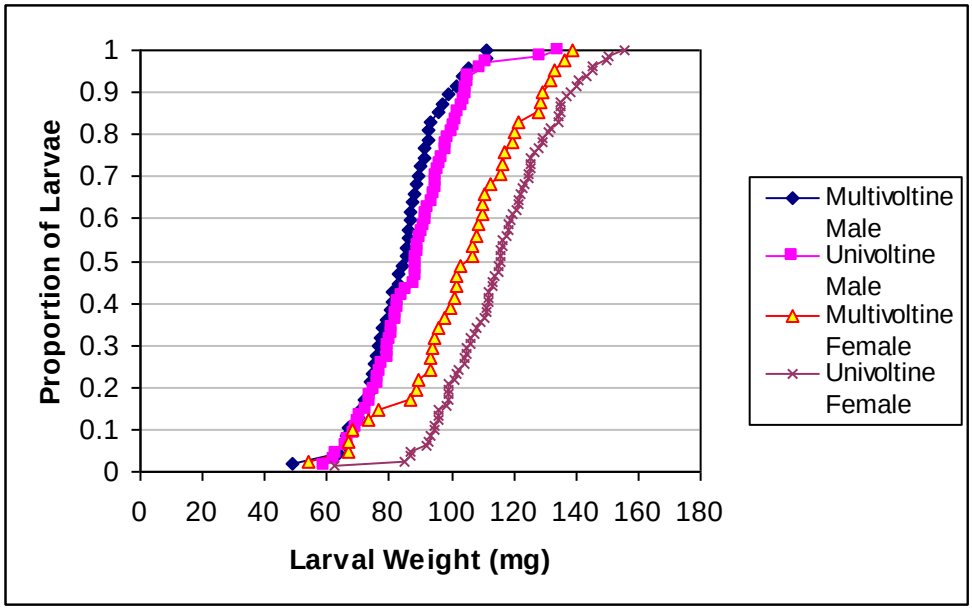
Appendix

SUPPORTING MATERIALS FOR CHAPTER 3

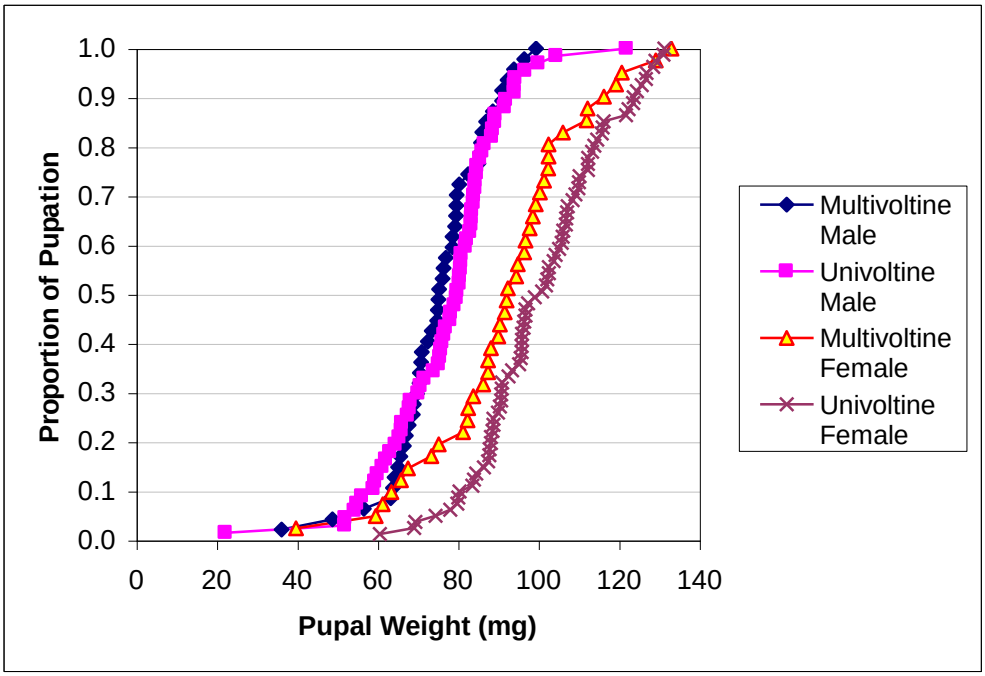
Appendix Figure 1: Cumulative Proportion of post-diapause univoltine and multivoltine European corn borer (*Ostrinia nubilalis*) males and females pupal weight collected in 2004 from Rock Spring, PA.



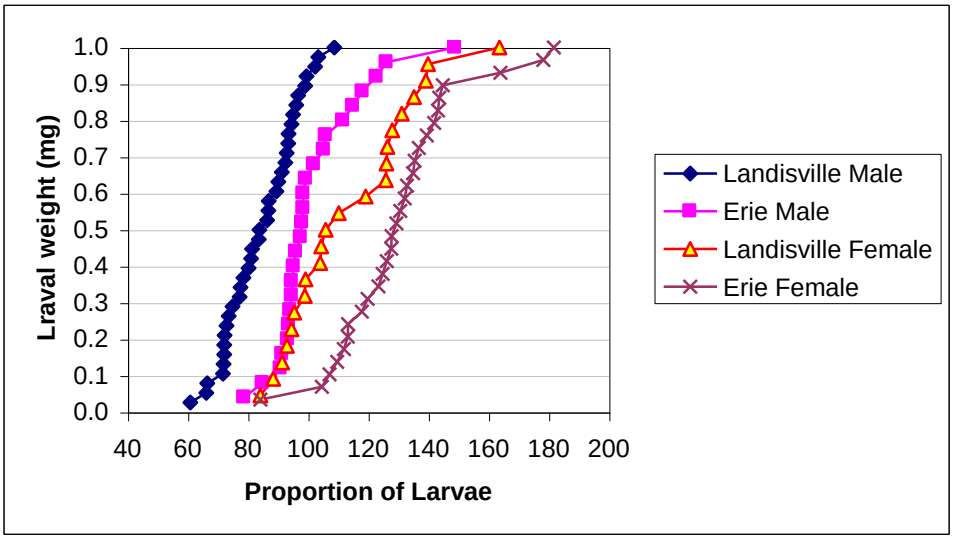
Appendix Figure 2: Cumulative Proportion of post-diapause univoltine and multivoltine European corn borer (*Ostrinia nubilalis*) males and females larval weight collected in 2005 from Rock Spring, PA.



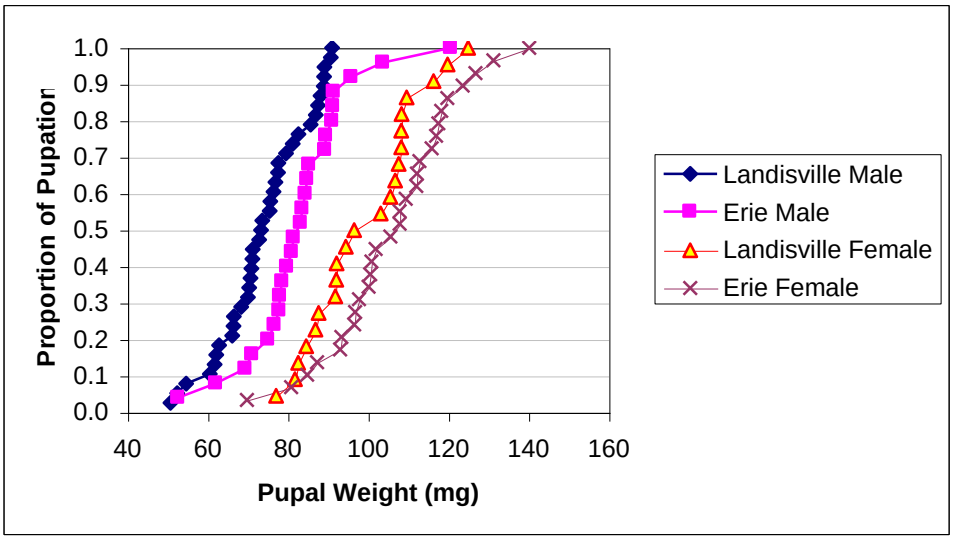
Appendix Figure 3: Cumulative Proportion of post-diapause univoltine and multivoltine European corn borer (*Ostrinia nubilalis*) males and females pupal weight collected in 2005 from Rock Spring, PA.



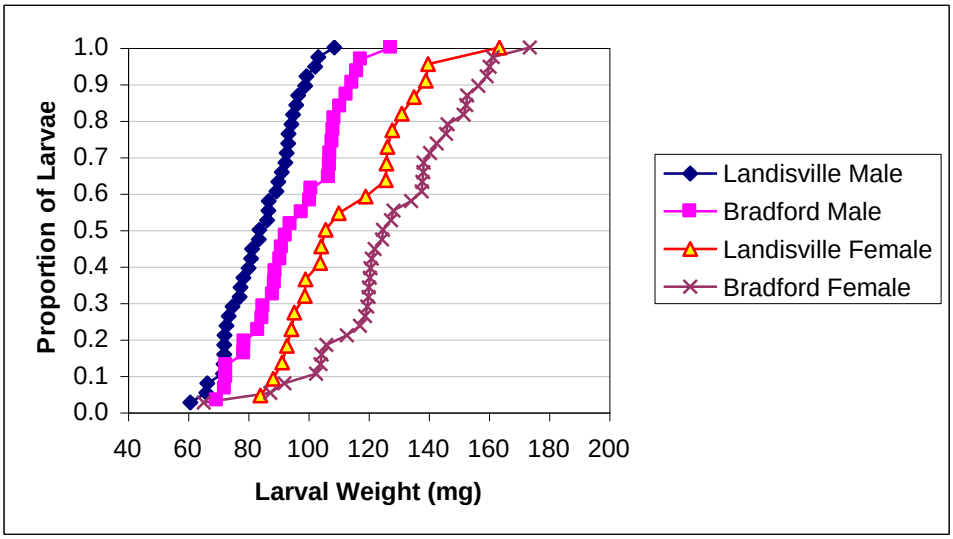
Appendix Figure 4: Cumulative proportion of larval weight of the post-diapause European corn borer (*Ostrinia nubilalis*) population collected from Landisville and Erie regions of multivoltine and univoltine ecotypes respectively in Pennsylvania.



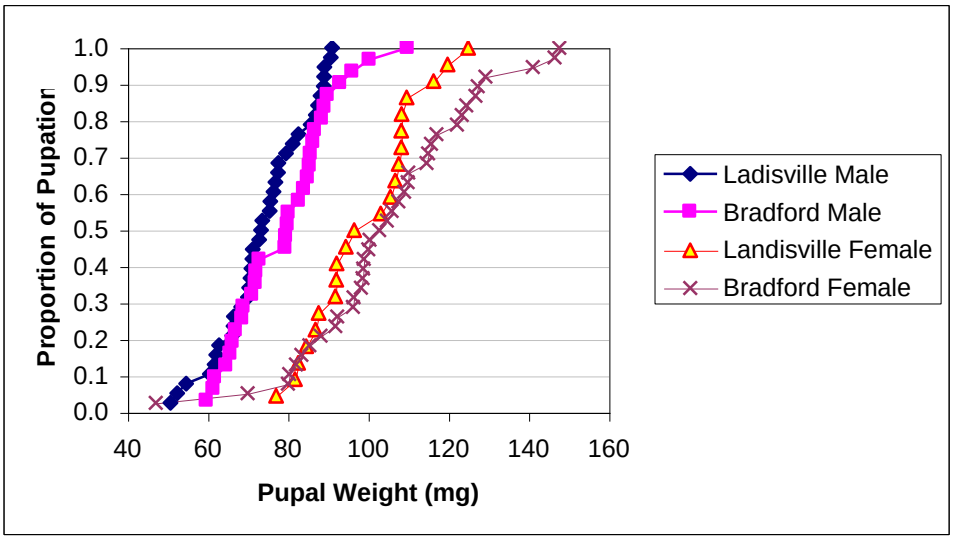
Appendix Figure 5: Cumulative proportion of pupal weight of the post-diapause European corn borer (*Ostrinia nubilalis*) population collected from Landisville and Erie regions of multivoltine and univoltine ecotypes respectively in Pennsylvania.



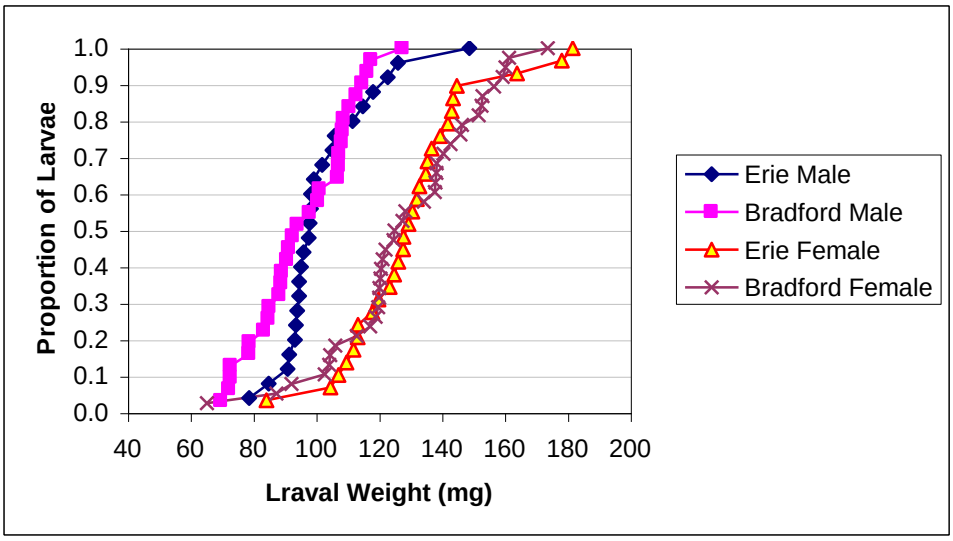
Appendix Figure 6: Cumulative proportion of larval weight of the post-diapause European corn borer (*Ostrinia nubilalis*) population collected from Landisville and Bradford regions of multivoltine and univoltine ecotypes respectively in Pennsylvania.



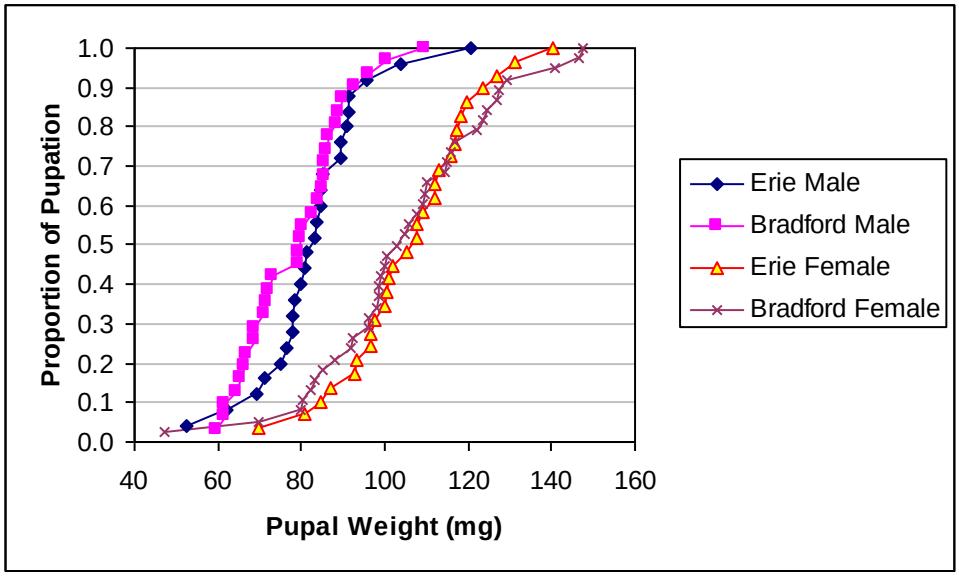
Appendix Figure 7: Cumulative proportion of pupal weight of the post-diapause European corn borer (*Ostrinia nubilalis*) population collected from Landisville and Bradford regions of multivoltine and univoltine ecotypes respectively in Pennsylvania.



Appendix Figure 8: Cumulative Proportion of larval weight of the post-diapause European corn borer (*Ostrinia nubilalis*) population collected from Erie and Bradford regions of univoltine ecotypes in Pennsylvania.



Appendix Figure 9: Cumulative Proportion of pupal weight of the post-diapause European corn borer (*Ostrinia nubilalis*) population collected from Erie and Bradford regions of univoltine ecotypes in Pennsylvania.



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