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Horizontal Transmission of Helicoverpa armigera Nucleopolyhedrovirus (HearNPV) in Soybean Fields Infested with Helicoverpa zea (Boddie)

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Horizontal Transmission of *Helicoverpa armigera* Nucleopolyhedrovirus (*Hear*NPV) in Soybean Fields Infested with *Helicoverpa zea* (Boddie)

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ABSTRACT

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) is a commercially available viral biopesticide that targets Heliothines, including *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), the most damaging pest of soybean (*Glycine max* (L.) Merrill) in the Mid-South. Previous formulations of *Hear*NPV have been well studied; however, no research has been published on current formulations. The first objective of this thesis was to assess the rate of horizontal transmission of *Hear*NPV in a soybean field infested with *H. zea* when *Hear*NPV was applied as a bio-insecticide, and to identify arthropods that are important obligate carriers in dissemination. *Hear*NPV spread 200 feet in 3 fields, and was present between 13 and 21 days post application. *Ceresa festina* (Hemiptera: Membracidae), *H. zea* larvae, and *Geocoris* spp. (Hemiptera: Geocoridae) were identified as important carriers, while spiders (Araneae) were determined to be suppressors of *Hear*NPV. The second objective was to identify arthropods in contact with infected larvae and determine their importance in dissemination. *Hear*NPV was found as far away as 200 feet by 3 days; however, only 2 samples were positive past 3 days. Several previously undocumented carriers were observed, including several families of Diptera and *Lygus lineolaris* (Hemiptera: Miridae). The third objective was to determine the ability of *Hear*NPV to kill each *H. zea* instar, and a second infestation. *Hear*NPV was successful in controlling 1^{st}-3^{rd} instars in 5 days. The second generation was controlled in 3.5 days. If applied to a soybean field as an insecticide when 1^{st}-3^{rd} instar *H. zea* populations are present, *Hear*NPV should be able to spread 200 feet, utilizing several over-sprayed arthropods such as *Ceresa festina*, and remain in the canopy from 3 to 21 days. Five days after the application, several arthropods can disseminate *Hear*NPV from liquefied to healthy larvae for 3 days before the majority of active *Hear*NPV leaves the canopy. If another generation of healthy larvae infest during this time period, the epizootic event should continue, thus repeating this cycle.

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CHAPTER ONE: LITERATURE REVIEW

Introduction:

The corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), is the most damaging pest of soybean (*Glycine max* (L.) Merrill) across the Mid-South (Musser et al. 2015a). *Helicoverpa zea* feeds on both vegetative and fruiting structures; however, the soybean plant is able to compensate for *H. zea* damage until the plant reaches the R4 and R5 growth stages (Mueller and Engroff 1980; Biever et al. 1983; Adams et al. 2015). Infestations that reach the economic threshold are usually controlled with an application of a synthetic insecticide; however, *H. zea* has developed resistance to many insecticides (Wolfenbarger et al. 1971, Sparks 1981, Abd-Elghafar et al. 1993, Kanga et al. 1996). It is likely that *H. zea* has developed resistance to pyrethroids, which leaves few effective classes of insecticides for control of *H. zea* (Musser et al. 2015b). Other tactics for controlling *H. zea* have been explored, such as the development of new insecticide classes and research on potential biocontrol agents such as *Helicoverpa armigera* Nucleopolyhedrovirus.

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV), a highly host-specific viral biopesticide, can be used to control *H. zea* populations with minimal off-target effects (Gröner 1986). Nucleopolyhedroviruses (NPV) are known for reaching high infective rates known as epizootic events, and in many cases can persist in the environment for extended periods of time (Young et al. 1977; Ignoffo et al. 1972; Ignoffo et al. 1976; Fuxa and Richter 2006; Fuxa 2008). *Hear*NPV is applied like other foliar insecticides and once ingested by larvae it multiplies within the host. After an infected host dies, it liquefies and becomes a source of the virus, which often results in horizontal transmission to other *H. zea* larvae present.

Several routes of horizontal transmission have been previously observed. Abiotic factors can contribute to the horizontal transmission of *Hear*NPV, with rapid transmission possible when ideal precipitation, wind, crop height, and soil conditions are present (Fuxa and Richter 2006; Fuxa and Richter 2001; Young 1990). However, this mode of transmission only occurs when physical forces act upon an existing bank of viral occlusion bodies that are present in the soil. Another route of transmission is from infected to uninfected larvae. This route of infection has been shown to occur in a multitude of ways including: contact or ingestion of frass from larvae that have been infected for over 3 days; deposition of viral occlusion bodies as sprayed larvae move around soybean plants; cannibalism of infected larvae by uninfected larvae; and through surface contamination as infected larvae liquefy or are wounded (Vasconcelos 1996; Ali et al. 1987a; Ali et al. 1987b).

Predators of *H. zea* and scavengers are also capable of spreading *Hear*NPV without being susceptible to the virus. Young and Yearian (1987) found detectable levels of NPV in a predator's frass up to ten days after that predator fed on an infected larva. Some arthropods have the potential to transport the virus by defecating adequate amounts of an NPV to cause infection (Lee and Fuxa 2000a; 2000b). Parasitoids are also a potential route of horizontal transmission via a contaminated ovipositor, emerging from a contaminated host, or being coated in the virus as an adult (Young and Yearian 1989; Young and Yearian 1990a).

Several routes of horizontal transmission have been observed in lab and cage studies; however, no study has attempted to analyze horizontal transmission in a naturally occurring infestation of *H. zea* in a soybean field. Studies have failed to identify both the rates of horizontal transmission in field infestations and the main factors of horizontal transmission for *Hear*NPV. Factors such as mobile, non-target arthropods could play a necessary role in horizontal

transmission, yet no in-field data are currently available. Without this information, accurately predicting horizontal transmission of *Hear*NPV within an *H. zea* infested soybean field could be difficult, if not impossible.

Soybean History and Cultivation:

Soybean (*Glycine max* (L.) Merrill) are a leguminous crop in the family Fabaceae. Linnaeus was the first to introduce the name *Glycine* in his book, *Genera Plantarum* in 1737; however, he described cultivated soybean as *Phaseolus max* and *Dolichos soja*. Later reclassifications by Bentham (1864, 1865) resulted in an arrangement similar to the current classification (Hymowitz and Newell 1981).

Soybean is believed to have been first domesticated in central or southern China 3,000 to 5,000 years ago, spreading along the Silk Road from the 1st century to the 15-16th century, reaching Europe by the 18th century, and first brought to North America by Samuel Bowen in 1765 when he planted soybean seeds at his plantation in Georgia (Morse 1950; Hymowitz 1970; Hymowitz and Harlan 1983). In 1851, the soybean was introduced to Illinois and the rest of the Corn Belt, but was grown primarily as forage until the 1920s (Hymowitz 1990). By 1941 soybean was planted primarily for the value of the bean in the U.S.

Soybean form a symbiotic relationship with *Bradyrhizobium japonicum* (Kirchner) Jordan (Rhizobiales: Bradyrhizobiaceae), a bacteria that results in nitrogen fixing root nodules (Cartter and Hartwig 1963). These nodules reduce atmospheric nitrogen to a form more readily available for the soybean plant to uptake. Soybean is a photoperiod-dependent short-day plant, where shortening day length triggers the soybean plant to begin flowering (Hamner 1944; Parker and Borthwick 1950). In Arkansas, seeds are planted between 1 April, after the last frost, until

late-June; however, planting during June begins to decrease yield potential (Beaver and Johnson 1981; Egli and Bruening 2000; Egli and Cornelius 2009). Egli and Cornelius (2009) found no added benefit for planting "ultra-early", planting between April and early-May, over planting from mid-May through late-May. Recently, there has been an increase in fields that are planted early or on time to avoid unfavorable photoperiods, late-season drought, disease, insect pressure, and harvest timings (USDA 1997; USDA 2010). Board (1985) found that the reduction in yield from planting at non-optimal planting dates was not due to a loss in the main stem yield components, but in the number of branches and branch nodes produced.

Once a soybean seed is planted, germination can take between 4 and 14 days depending on environmental conditions and variety (Cartter and Hartwig 1963). As the soybean plant develops, new main-stem nodes develop approximately every 4 days. Fehr et al. (1971) described each new node as a new vegetative growth stage, which are categorized by V1-Vn, with "n" being the number of nodes present. Reproductive stages are described as R1-R8, starting at initial flowering (R1) and ending with harvest maturity (R8) (Fehr et al. 1971). Soybean varieties are either determinate or indeterminate (Beaver and Johnson 1981). Determinate varieties stop vegetative growth and production of nodes on the main stem soon after flowering starts, but will continue producing nodes on branches until the 5^{th} reproductive stage. Indeterminate varieties continue producing nodes on the main stem until the 5^{th} reproductive stage. Both growth patterns have comparable bloom times, with flowering taking 3-6 weeks (Beaver and Johnson 1981). Flowering is followed by pod development (R3/R4), bean fill (R5/R6), and physiological (R7) and harvest (R8) maturity.

Helicoverpa zea (Boddie):

Helicoverpa zea (Boddie) (cotton bollworm, corn earworm, or tomato fruitworm) is a major pest in several row crops including corn, cotton, soybean, and sorghum (Quaintance and Brues 1905). The larvae of *H. zea* are polyphagous, with hosts in 22 plant families (Quaintance and Brues 1905). Host plants for *H. zea* range from weedy species such as *Ipomoea* spp. (L.) (Solanales: Convolvulaceae) to economically important crops such as *Gossypium* (L.) spp. (Malvales: Malvaceae), *Hibiscus esculentus* L. (Malvales: Malvaceae), *Zea mays* L. (Poales: Poaceae), *Sorghum bicolor* (L.) Moench (Poales: Poaceae), *Saccharum officinarum* L. (Poales: Poaceae), *Trillium* L. spp. (Liliales: Melanthiaceae), *Avena sativa* L. (Poales: Poaceae), *Oryza sativa* L. (Poales: Poaceae), and *Vitis* L. spp. (Vitales: Vitaceae). Adult female *H. zea* prefer to oviposit in corn on new silks over tobacco, cotton, or soybeans, but can oviposit on any host plant present (Johnson et al. 1975). Fitt (1989) stated an annual estimated cost of damage by *H. zea* in the United States was over $1 billion for all crops. From 2011 to 2014, *H. zea* was the most damaging pest in soybean across the Mid-south, and the second most damaging pest in 2009, 2010, and 2015 (Musser et al. 2010; 2011; 2012; 2013; 2014; 2015a; 2016). In Arkansas, *H. zea* was the most damaging pest from 2010 to 2013 and 2015; in 2009 and 2014, it was the second most damaging pest. The estimated economic impact of the corn earworm in soybean from 2009 to 2015 was between $24 and $156 million in Arkansas alone, and on average was approximately $76 million (Musser et al. 2010; 2011; 2012; 2013; 2014; 2015a; 2016).

Seasonal Distribution

Helicoverpa zea is widely distributed, and occurs throughout most of the world from the 50°N to the 50°S latitudinal parallels (Quaintance and Brues 1905). The original geographic

range of this pest is not known; however, humans are believed to have been an important factor in their dispersal. The first record of *H. zea* as a pest is in North America in 1820, in cotton, and by 1841 *H. zea* had become a prominent pest in cotton and corn in the southern United States (Quaintance and Brues 1905). Adults emerge in late spring to early summer, and begin laying eggs on suitable hosts three days after emerging (Ditman and Cory 1931). The adults are primarily nocturnal, and engage in both short-range and migratory movement (Fitt 1989). Short-range movement is the behavior of feeding, oviposition, mating, and finding shelter, while migratory movement occurs when moving long distances at high altitudes. The migration patterns of *H. zea* are highly variable and dependent on wind patterns, with the first generation typically emerging in areas south of Interstate 40 in Arkansas, including the southern Arkansas Delta, around March or April (Sandstrom et al. 2007). Hendrix et al. (1987) trapped adult male *H. zea* moths in Arkansas, and identified two types of pollen that are not found in Arkansas, with the closest distribution being from south central and south Texas. This indicates that *H. zea* adults migrate from overwintering sites in south Texas to Arkansas. Subsequent generations are capable of reaching as far north as Canada (Sandstrom et al. 2007).

Morphology and Life Cycle

Helicoverpa zea overwinter as pupae in the soil in areas where the temperature does not drop to 0°F for at least four consecutive days; this includes the majority of Arkansas, with only the northeastern corner of Arkansas occasionally not being suitable for overwintering (Sandstrom et al. 2007). The pupae emerge as adults from the soil in early spring, forming the first generation. These adults feed on nectar and begin to oviposit on wild hosts 3 days after emerging (Capinera 2001). The second or third generation moves to cultivated hosts (Barber

1937). *Helicoverpa zea* eggs are nearly spherical and faintly yellow in color, and are approximately 0.02in in diameter (Ditman and Cory 1931; Neunzig 1964, 1969). Adult female moths lay eggs individually on host plants suitable for the development of their progeny, and can lay up to 35 eggs per day and 500 to 3,000 eggs over their lifetime (Ditman and Cory 1931; Fitt 1989; Eckel et al. 1992). The egg hatches approximately 3 days after oviposition, and the larvae of *H. zea* normally undergo six instars before pupation (Quanitance and Brues 1905; Hardwick 1965). Each instar is readily distinguishable from the other, with descriptions published by Ditman and Cory (1931), Neunzig (1964), and Quantance and Brues (1905). The *H. zea* larvae have a cylindrical body with short black thoracic legs and four pairs of prolegs along the abdomen (Quaintance and Brues 1905). Dark microspines are located along the body, which can be used to distinguish *H. zea* from other common lepidopteran pests (Hardwick 1965). Larval color is not a good identification characteristic because it is highly variable in this pest. The development time of the larval stage varies based on the temperature and nutritional composition of the diet the larva receives (Butler 1976). At 89.6°F, a larva takes 9-20 days to develop, and once fully developed, will drop off the plant and burrow 5-10 centimeters into the soil, forming a pupal chamber (Quaintance and Brues 1905; Capinera 2001). The pupal stage lasts approximately 13 days, with adults emerging and migrating to areas where their host plants are present (Hardwick 1965; Eckel et al. 1992). Complete development, from egg to adult, under ideal temperature, food, and rainfall will take approximately 30 days. Typically 4 to 6 generations of corn earworm occur during a year within the United States (Quaintance and Brues 1905; Ditman and Cory 1931). An adult, depending on environmental factors, generally lives 5-10 days, and is primarily active at night and rests under crop canopies or other shaded areas during the day (Quaintance and Brues 1905; Ditman and Cory 1931).

Soybean Damage

The distribution of *H. zea* within a soybean canopy varies based on the instar (Eckel et al. 1992). Small larvae prefer to feed on rolled leaves and flowers, probably because of the nutritional content and the protection offered by these sites. Larger larvae can be found anywhere on the plant, feeding on leaves, stems, blooms, or pods, with each larva capable of damaging approximately six pods over their lifetime (Mueller and Engroff 1980; Biever et al. 1983; Eckel et al. 1992). Sixth instar larvae are responsible for more damage than the 4th and 5th instar combined (McWilliams 1983). Defoliation at bloom caused by *H. zea* feeding and/or other factors can result in yield loss when two thirds or more of the leaves are removed, but once the bean is half full (R5.5) the yield loss is less pronounced (Begum and Eden 1965). However, the most extensive damage caused by *H. zea* occurs when late 3rd-instar larvae feed on R4 soybean, which would typically result from oviposition when the soybean plants were in full bloom (R2) (McWilliams 1983). A six day delay in infestation could result in as much as a 50% reduction in damage due to the later developmental stage of the soybean when the larvae reach the 3rd instar. As the soybean plant develops past bloom it becomes more unsuitable to neonates and early instar *H. zea* larvae (Terry et al. 1987). Mueller and Engroff (1980) determined that soybean compensation plays a vital role in offsetting *H. zea* damage. The study used infestation levels as high as 20 larvae per row foot and concluded that when blooms were consumed or pods or beans were damaged the plant was able to completely compensate, either by re-blooming or enlarging and completely developing the remaining beans. This correlation between level of injury and growth stage and the level of infestation was also observed by Biever et al. (1983) with the yield loss from *H. zea* damage occurring primarily around R4 and R5 due to the larvae feeding on

young developing pods. This coincides with a study conducted by Thomas et al. (1976) where it was found that the R3 and R4 reproductive stages are the last stages where the plant can compensate for yield loss from insect damage. Intense feeding and defoliation on soybean plants does not lead to compensation through increased vegetative growth as would be seen in cotton plants, and yield was not reduced in soybean until R4 or R5. However, significant delays in maturity resulted from fruit loss at R5.5 (Adams et al. 2015).

Economic Threshold

The economic threshold for *H. zea* has been changed several times in Arkansas since it was established in 1965 at 3 larvae per 10 row feet. Mueller and Engroff (1980) stated that the original threshold of 3 larva/10 row ft was changed to 9 larvae/10 row ft in 1967, 20 larvae/10 row ft in 1972, 30 larvae/10 row ft in 1978, and 40 larvae/10 row ft in 1981. However several studies have pointed out that *H. zea* are defoliators and pod feeders, and depending on the growth stage of the plant and infestation levels, yield loss might not occur from feeding. When yield loss does occur from *H. zea* feeding, it does not reduce the bean quality (Begum and Eden 1965; Thomas et al. 1974; Thomas et al. 1976; Joshi 1980; Biever et al. 1983; McWilliams 1983; Eckel et al. 1992). To reduce some of the inconsistencies, a dynamic threshold was developed and introduced through collaboration from entomologists in the Mid-South. Before bloom soybeans should be treated when 40% defoliation occurs, but after bloom is more dynamic. The market value of the soybean, the cost of control, and the infestation level is utilized to determine what the threshold should be for the field that is infested (Adams et al. 2015; Adams et al. 2016b). Tables summarizing this threshold can be found in the Insecticide Recommendations for Arkansas (Studebaker et al. 2017).

Control Tactics:

Cultural Control

There are several practical methods that can be implemented in most integrated pest management (IPM) systems that will reduce the possibility of having a damaging infestation of *H. zea*. One of the simplest methods of cultural control to implement is early planting. A study conducted by Nault et al. (1992) showed *H. zea* preferred feeding on leaves from younger plants. A separate study conducted by Joshi (1980) showed delaying planting increases pod damage by *H. zea* due to populations increasing throughout the season. By planting before May 28th and using early maturing varieties, exposure can be minimized, while planting after May 28th (including double-cropped fields) are more susceptible to infestations. In addition, Joshi (1980) found that some cultivars were less prone to *H. zea* damage through antibiosis, non-preference, and tolerance, implying that cultivar choice could also be a form of cultural control. Tillage can be used to reduce overwintering populations of *H. zea*; however, this only results in local suppression, and as the adult is highly mobile this practice is not practical (Barber and Dicke 1937; Fife and Graham 1966). Another cultural control practice is to plant on narrower row spacing (Oplinger and Philbrook 1992; Elmore 1998). This allows the canopy to close sooner which reduces stress on the plant, allowing for quicker maturation times. However, intraspecific competition is a major yield limiting factor. Alston et al. (1991) found that fields with higher weed densities also reduced *H. zea* populations even with the added stress from the weeds, possibly because of the canopy closure. While this is not something to be desired in a field, it helps show the importance of canopy closure in managing *H. zea* populations.

Insecticidal Control

Once an infestation reaches the economic threshold the primary method for the control of *H. zea* has been the use of chemical insecticides. Over time, populations of *H. zea* have become resistant to several classes of insecticides, including chlorinated hydrocarbons, organophosphates, and carbamates (Brazzel 1963; Graves et al. 1963; Lingren and Bryan 1965; Adkisson and Nemec 1967; Carter and Phillips 1968; Plapp 1971; Wolfenbarger et al. 1971; Lentz et al. 1974; Sparks 1981; Abd-Elghafar et al. 1993; Kanga et al. 1996). For several years the most used insecticide class has been the pyrethroids, which are cheap and relatively non-toxic to humans (Myamoto 1976; Abd-Elghfar et al. 1993; Musser 2015b). However, *H. zea* is beginning to develop resistance to this class, with some strains in Arkansas showing 5- and 10-fold resistance compared to susceptible strains (Abd-Elghafar et al. 1993; Kanga et al. 1996; Musser et. al. 2015b). In 2008 a novel class of insecticides, known as diamides, were marketed; several insecticides in this class provide excellent control, but are generally more expensive than pyrethroids (Adams et al. 2016a). In 2016 the EPA cancelled the registration of flubendiamide, a diamide insecticide, and it was removed from the market due to concerns about detrimental effects on benthic organisms based on a model used by the EPA (EPA 2016). Due to the resistance potential of *H. zea* there is a need for new insecticides that are effective, economical, and not harmful to humans or the environment.

Biological Control

Helicoverpa zea has numerous natural enemies including both generalist and specialist predators and parasitoids. In some fields the levels of these natural predators and parasitoids can delay or prevent the development of major infestations. Barber (1942) found certain birds were predating

on *H. zea* larvae in corn ears. *Nabis* spp., particularly *Nabis roseipennis* Reuter (Hemiptera: Nabidae), are the predominant predator of *H. zea* eggs and larvae in soybeans, but there are several other egg predators such as *Geocoris punctipes* (Say) (Hemiptera: Geocoridae), *Coleomegilla maculate* DeGeer (Coleoptera: Coccinellidae), *Orius insidiosus* Say (Hemiptera: Anthocoridae), *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), and *Clubiona abbotii* Koch (Araneae) (Oatman 1966; Pfannenstiel and Yeargan 2002). In a study conducted by Sansone and Smith, Jr. (2001), the most prevalent predator was *O. tristicolor* (White) (Hemiptera: Anthocoridae) and *O. insidiosus* Say (Hemiptera: Anthocoridae), with *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), *Scymnus* spp. (Coleoptera: Coccinellidae), and multiple species of spiders (Araneae) also present. McPherson et al. (1982) found a number of predators in soybean fields: *Nabis* spp., *G. punctipes*, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), *Stiretrus anchorago* (Fabricius) (Hemiptera: Pentatomidae), *H. tredecimpunctata* (L.) (Coleoptera: Coccinellidae), *H. convergens*, *Zelus* spp. (Hemiptera: Reduviidae), *Sinea* spp. (Hemiptera: Reduviidae), *Arilus* spp. (Hemiptera: Reduviidae), *Chrysopa* spp. (Neuroptera: Chrysopidae), and mantids (Mantodea).

The main parasitoids of *H. zea* are *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) and *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) (Lewis and Brazzel 1968; Sansone and Smith 2001). Several parasitoids are known to attack *H. zea* including: *T. pretiosum* Riley (Hymenoptera: Trichogrammatidae), *T. exiguum* Pinto & Platner (Hymenoptera: Trichogrammatidae), *T. pretiosum* Riley (Hymenoptera: Trichogrammatidae), *Chelonus texanus* Cresson (Hymenoptera: Braconidae), *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), *Elasmus setosiscutellatus* Crawford (Hymenoptera: Eulophidae), a pupal parasitoid, *Ichneumon promissorius* (Erichson) (Hymenoptera: Ichneumonidae), *Archytas*

marmoratus (Townsend) (Diptera: Tachinidae), and *Eucelatoria bryani* Sabrosky (Diptera: Tachinidae) (Oatman 1966; Lewis and Brazzel 1968; Martin et al. 1976; Kogan et al. 1989; Steward et al. 1990; Carpenter et al. 1994). Aphelinidae, Chalcididae, and Platygastridae also have members known to parasitize *H. zea*.

There are also several nematode species that are known to attack *H. zea* pre-pupae and pupae such as *Steinernema riobrave* (Rhabditida: Steinernematidae), *S. carpocapsae*, *Chromonema heliothidis* (Rhabditida: Steinernematidae), and *Chroniodiplogaster aerivora* (Cobb) (Rhabditida: Diplogasteridae) (Khan et al. 1976; Purcell et al. 1992; Steinkraus et al. 1993; Cabanillas and Raulston 1996).

Viruses in Insects:

The first virus reported in insects was described as jaundice of the silkworm by Nysten in 1808. In 1856, Maestri and Cornalia separately observed crystalline bodies in infected silkworm cell nuclei using a microscope, and by 1894, these structures were named polyhedral granules by Bolle and were thought to be proteinaceous (Smith 1973). In 1918, Acqua provided proof that jaundice of the silkworm was viral and was protected by inclusion bodies, and the pathogen has since been classified as a baculovirus (Glaser and Stanley 1943; Benz 1986).

There are more than 1100 species of viruses that infect invertebrates, with the majority infecting insects (Adams 1991). Most of these insect-infecting viruses are found in the family Baculoviridae; however, there are entomopathogenic viruses in fifteen other viral families: Ascoviridae, Birnaviridae, Caliciviridae, Dicistroviridae, Iflaviridae, Iridoviridae, Nodaviridae, Nudiviridae, Parvoviridae, Picornaviridae, Polydnaviridae, Poxviridae, Reoviridae, Rhabdoviridae, and Tetraviridae (Hunter-Fujita et al. 1998). Insect viruses are classified based

on genome composition and size, virus shape and size, and presence or absence of an envelope or occlusion body (Boucias and Pendland 1998). Viral genomes are comprised of either RNA or DNA ranging in size from 1 to 300 kilobase-pairs (kb) arrayed in linear or circular structures of double or single strands (Boucias and Pendland 1998). This genome, coupled with any structural proteins or enzymes unique to the virus, is the nucleocapsid. The nucleocapsid can be non-enveloped, naked virus, or enveloped, surrounded by a membrane of glycoproteins. This is known as a virion.

Ascoviruses are large, enveloped nucleocapsids with structural proteins arranged in either reniform or bacilliform (Federici 1983; Tanada and Kaya 1993; Boucias and Pendland 1998). The viral genome consists of linear double-stranded DNA approximately 170 kb in length. These viruses appear to only affect noctuid species, killing the host larvae when infected cell nuclei enlarge and then break apart (Federici 1983). Ascoviruses are transmitted primarily through infected parasitic wasps, and are capable of outcompeting the developing parasitoid.

Birnaviruses are approximately 60 nm in diameter, non-enveloped, and the structural proteins are arranged in an icosahedral shape (Tanada and Kaya 1993; Boucias and Pendland 1998). The viral genome is a two-segmented, double-stranded RNA genome. The only identified virus of insects in this family is *Drosophila* X virus.

Caliciviruses are a small virus, approximately 35 nm in diameter (Tanada and Kaya 1993; Boucias and Pendland 1998). The viral structure does not contain an envelope, and the structural proteins are arranged in an icosahedral form with cup-shaped depressions. The genome is single-stranded RNA, and the only known insect virus from this family is Chronic Stunt Virus, which affects the navel orangeworm, *Amyelois transitella* (Kellen and Hoffmann 1981). This virus kills early instars, and results in a chronic infection in later instar larvae which can reduce fecundity.

Two newly formed families of viruses that contain several honeybee viruses are Dicistroviridae and Iflaviridae. Dicistroviruses are naked single stranded RNA viruses. Acute Bee Paralysis Virus, Kashmir Virus, and Black Queen Cell Virus belong to this family. Iflaviruses are infectious flacherie viruses. Sacbrood Virus and Deformed Wing Virus belong to this family.

Iridoviruses are large-genome viruses, containing around 225 kb of linear, double-stranded DNA in an icosahedral structure (Tinsley and Kelly 1970; Tanada and Kaya 1993; Boucias and Pendland 1998). It is unknown how these viruses infect healthy hosts because oral infection and epizootics are rare. However, this family does cause mortality in early instars. Once an infection is established and the virus is replicating, an iridescent hue can be observed from the host due to the virion alignment in the host cell cytoplasm.

Nodaviruses contain an enveloped nucleocapsid in an icosahedral structure approximately 30 nm in diameter (Tanada and Kaya 1993; Boucias and Pendland 1998). They were first isolated from mosquitoes in Japan (Moore et al. 1985). The genome consists of two segments of single-stranded RNA. These viruses are all insect pathogens, infecting mainly Lepidoptera and Coleoptera. The Black Beetle Virus was the first insect viral genome to be completely sequenced.

Parvoviruses are small, naked nucleocapsids with structural proteins arranged in an icosahedral structure (Tanada and Kaya 1993; Boucias and Pendland 1998). The genome consists of both positive- and negative-stranded DNA that are separately encapsulated and approximately 4 to 5 kb (Newman and Brown 1977). These viruses mainly affect Lepidoptera, but can occur in Diptera, Odonata, and Orthoptera. These viruses result in either acute infection and mortality or chronic infection with delayed mortality.

Picornaviruses are small, single-stranded RNA viruses that are naked, with structural proteins exhibiting icosahedral symmetry approximately 30 nm in diameter (Tanada and Kaya 1993; Boucias and Pendland 1998). Their nucleocapsid is comprised of only four polypeptides. There are over 30 picornaviruses that are suspected to infect insects; however, few of these have been confirmed (Moore et al. 1985).

Polydnaviruses contain a complex genome of multiple super-helical DNA strands of varying size, between 2 and 28 kb (Stoltz et al. 1984; Tanada and Kaya 1993; Boucias and Pendland 1998). These viruses that affect insects only replicate in the calyx of parasitic Hymenoptera (Cook and Stoltz 1983). They have a symbiotic relationship with the species they utilize as a host, with all females of that species being infected. When the female lays an egg, she injects the virus as well, which may suppress the immune response and aid in development of the hymenopteran progeny.

Rhabdoviruses are bullet-shaped virions, containing single-stranded RNA (Tanada and Kaya 1993; Boucias and Pendland 1998). The only Rhabdovirus restricted to insects is the Sigma Virus, affecting 10% of the natural populations of *Drosophila*, and is only transmitted vertically. This virus causes *Drosophila* to die when exposed to CO_2 at normally sub-lethal doses.

Tetraviruses only affect Lepidoptera, and are naked viruses approximately 35 to 40 nm in diameter (Tanada and Kaya 1993; Boucias and Pendland 1998). This family is comprised of both one- and two-segmented single-stranded RNA genomes. Tetraviridae contains only 19 species of viruses, but currently little is known about the biology or life cycle of these viruses.

Reoviruses classified in the subfamily cytoplasmic polyhedrosis viruses (CPV) have only been isolated from arthropods, and have been recorded in 250 insect species (Xeros 1952; Tanada and Kaya 1993; Boucias and Pendland 1998). These viruses have an occlusion body,

which is a protein structure surrounding the virion. These viruses replicate in the midgut epithelial cells, where large numbers of occlusion bodies are produced, forming a crystallogenic matrix (Arnott et al. 1968). CPVs cause chronic infections resulting in malformation and reduced fecundity. Their genome consists of 10 segments of double-stranded RNA, and the naked nucleocapsid is arranged in icosahedral symmetry. Unlike other reoviruses, CPVs have only a single capsid shell rather than a double. However, they do have 12 spikes on the capsid, as seen with other reoviruses.

Entomopoxviruses (EPV), classified as a subfamily of Poxviridae, affect around 60 insect species (Granados and Roberts 1970; Tanada and Kaya 1993; Boucias and Pendland 1998). They are ovoid in shape, and with the exception of hymenopteran EPVs, the nucleocapsid is contained within a spheroidal OB called spherules or spheroids (Weiser 1969). These viruses replicate in the cytoplasm of infected fat body cells, however, some infections are systemic. Their genomes are large linear double-stranded DNA.

Baculoviridae:

The Baculoviridae viral family contains more viruses of insects currently identified than any other viral family (Herniou and Jehle 2007). They are rod-shaped with enveloped viruses containing a circular double-stranded DNA genome, surrounded by a proteinaceous inclusion body (Bilimoria 1986). There are four genera within Baculoviridae: *Alphabaculovirus* (lepidopteran-specific Nucleopolyhedroviruses), *Betabaculovirus* (lepidopteran-specific Granuloviruses), *Gammabaculovirus* (hymenopteran-specific Nucleopolyhedroviruses), and *Deltabaculovirus* (dipteran-specific Nucleopolyhedroviruses) (Jehle et al. 2006). Prior to this reclassification, Baculoviruses contained a genus of non-occluded viruses, however these were

placed in a new family, Nudiviridae, at the 6th International Committee on Taxonomy of Viruses (ICTV) (Murphy et al. 1995). Granuloviruses contain a single virion packaged in a single ovule inclusion body (Bilimoria 1991). Nucleopolyhedroviruses (NPVs) contain several virions occluded in a single polyhedral inclusion body. NPVs can be further divided into single-nucleocapsid NPVs (SNPV) and multi-nucleocapsid NPVs (MNPV). Single-nucleocapsid NPVs contain a single nucleocapsid per envelope, while MNPVs contain several nucleocapsids per envelope (Bilimoria 1991). The occlusion body protects the virion from degradation during extended exposure to the environment and disintegrates once in the midgut of the host (Bilimoria 1991). The virions are then released and infect the gut epithelial cells where the production of budded virus begins. All budded viruses contain one nucleocapsid per virion enveloped by the nuclear or plasma membrane of the host cell. The budded viruses are transferred to the fat body through the hemolymph (Hunter-Fujita et al. 1998). Once in the fat body, millions of occlusion bodies are formed as the lethargic larvae disintegrates, releasing the occlusion bodies into the environment (Boucias and Pendland 1998). Baculoviruses have been used as biopesticides in forestry, orchards, and row crops since the early 1900s with a fair amount of success; however, the slow kill and degradation due to environmental conditions have limited their uses (Inceoglu et al. 2006).

***Helicoverpa armigera* Nucleopolyhedrovirus:**

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) is a virus in the family Baculoviridae, in the genus *Alphabaculovirus*. This virus has been known by several names, being isolated from *Helicoverpa armigera* and *Helicoverpa zea.* Originally, this virus was called either *Heliothis* NPV or *Heliothis zea* NPV, first reported in the ICTV 5th report (Francki et al.

1991). Then, in 1995 it was renamed to *Helicoverpa zea* SNPV in the ICTV 6th report (Murphy et al. 1995). In 2002, *Helicoverpa armigera* NPV was added to the genus *Nucleopolyhedrovirus,* and in 2008, the genus *Nucleopolyhedrovirus* was abolished and the current classification was established, with *Helicoverpa armigera* NPV and *Helicoverpa zea* SNPV moving to the genus *Alphabaculovirus* (Mayo 2002; Jehle et al 2006). Finally, in 2014, *Helicoverpa armigera* NPV and *Helicoverpa zea* NPV merged to *Helicoverpa armigera* NPV after genomic research showed them to be the same virus (Rowley et al. 2011; Harrison et al. 2014). *Helicoverpa armigera* Nucleopolyhedrovirus (*Hear*NPV) is a large, occluded, double-stranded DNA virus. Once the occlusion bodies of *Hear*NPV is ingested, the alkaline environment of the midgut dissolves the occlusion bodies, releasing the occlusion-derived virus (Bilimoria 1991). The host peritrophic membrane is in a constant state of regeneration from epithelial cells due to ingested food and is the initial defense of the host (Wigglesworth 1965). Once the virus has crossed the peritrophic membrane, the virus enters the midgut cells where the virus replicates, producing a budded virus which does not have an occlusion body for protection. During the early phase, budded virus is produced in the midgut cells and infects tracheal epidermal cells and then moves to hemocytes and fat bodies (Harrap and Robertson 1968; Hunter-Fujita et al. 1998). During the later phase of viral replication, the virus produces occlusion-derived virus inside the occlusion bodies. The larval host migrates to the upper leaves of the host plant, and liquefaction occurs when the host dies, releasing the occlusion bodies into the environment.

*Hear*NPV is currently commercially available, and only affects *Helicoverpa* species (Young and McNew 1994). This virus offers several advantages: it is cost effective; highly specific in its target thereby leaving the beneficial complex intact; can be applied like an insecticide; and has the potential to create an epizootic event (Gröner 1986; Fuxa and Tanada

1987; Inceoglu et al. 2006). The application can be spread by abiotic as well as biotic means, and can remain viable in soils for years (Young and Yearian 1990a, 1990b; Fuxa et al 2001). Evaluation studies were performed to determine the efficacy of using *Hear*NPV in both field and laboratory settings (Stacey et al. 1977a, 1977b, 1977c; Luttrell et al. 1982a). These studies concluded that the virus is most effective at killing earlier instars and that higher concentration dosages resulted in higher mortality rates (Stacey et al. 1979; Luttrell et al. 1982a; Yearian and Lorenz 1983).

Efficacy and Persistence of *Helicoverpa armigera* Nucleopolyhedrovirus:

One disadvantage of using *Hear*NPV as a biological insecticide is the potential for a low rate of persistence after application due to inactivation, especially when compared with chemical insecticides. This potential for quick degradation by environmental factors is one reason farmers have not readily implemented *Hear*NPV into their application rotations (Ignoffo et al. 1972; Young and Yearian 1974; Yearian and Young 1974; Ignoffo et al. 1976; McLeod et al. 1977). Several studies have been conducted looking at the stability and inactivation of the virus as a result of dispersal methods, ultraviolet radiation, dew pH, temperature, and distribution on the plant (Ignoffo et al. 1972; Young and Yearian 1974; Yearian and Young 1974; Ignoffo et al. 1976; Young et al. 1977; McLeod et al. 1977; Young 1990). McLeod et al. (1977) found *Hear*NPV to be inactivated by ultraviolet light and with an increase in temperature in the presence of ultraviolet light. Young and Yearian (1974) stated that the majority of inactivation occurred during daylight hours, therefore suggesting sunlight as the primary source of deactivation. Ignoffo et al. (1972) found the half-life of *Hear*NPV to be around 0.9 days. Yearian and Young (1974) found *Hear*NPV degraded quickly in the upper canopy, but was active in the

middle and lower canopy for up to 96 hours after the application. Applications on soybeans retain approximately 50% activity after 24 hours and 33% after 48 hours in fields with continuous exposure to ultraviolet light (Yearian and Young 1974). McLeod et al. (1977) also found the drying of cotton dew at a pH of 9.3 resulted in inactivation of *Hear*NPV, but dew at a pH of 7.4 or 8.8 had no effect. This was supported by Young et al. (1977) where it was determined that the virus suspended in dew was not deactivated, cotton dew deactivated the virus as it dried, and soybean dew had no effect. Studies conducted to reduce the inactivation of *Hear*NPV have resulted in new viruses and viral formulations that are more persistent when exposed to ultraviolet light (Ignoffo et al.1972; Ignoffo et al. 1976; Jeyarani et al. 2013).

Another disadvantage of the virus is the relatively slow time to host mortality. *Hear*NPV is effective at killing the early instars of *H. zea*; however, it is not as fast acting as an insecticide, taking between 3-8 days to kill the inoculated host, and does not perform with satisfaction on larvae larger than the third instar (Luttrell et al. 1982a). Studies were conducted to explore the addition of adjuvants that stimulated larval feeding, which would increase the amount of viral occlusion bodies the larva ingested (Stacey et al. 1977a; Luttrell et al. 1982b; Luttrell et al. 1983). Conclusions from these studies varied from increased mortality (Luttrell 1982b) to no change in dosage-mortality, ultraviolet degradation, or spray droplet properties, although larval feeding was still increased (Luttrell et al. 1983). These conclusions indicated that adjuvants increased efficacy by increasing the larval feeding rates. When adjuvants were added to the virus there did not seem to be an increase in crop yield except when using invert sugar, which is when sucrose undergoes hydrolysis by the invertase enzyme (Stacey et al. 1977a). Tank-mixes were studied in order to determine if there were any synergistic relationships between the virus and chemical insecticides, utilizing both laboratory and field studies (Pieters et al. 1978; Luttrell et

al. 1979; Mohamed et al. 1983). The study conducted by Pieters et al. (1978) was under low pressure and found the virus to be just as effective as both tank-mixes and the chemical insecticide alone. Luttrell et al. (1979) found similar results, showing no significant differences between tank-mixes and the virus alone. The only *Hear*NPV-insecticide mix that was found to be synergistic was *Hear*NPV-chlordimeform (Formamidine), and all others were antagonistic (Mohamed et al. 1983). While this biological insecticide still has a lower mortality rate compared with a chemical insecticide, a proper timing threshold could be determined in order to make *Hear*NPV a more efficient insecticide like the system proposed by Flusche et al. (1986). They proposed a threshold based on assigning feeding units to each larval instar and determining pest population larval instars to see if *Hear*NPV could be successfully implemented.

Host plants influence the epizootic potential of *Hear*NPV, with more occlusion bodies being produced from larvae that fed on vegetative rather than reproductive tissue (Ali et. al 2002). There is also a difference observed in efficacy depending on the crop, as *Hear*NPV had a higher rate of mortality in soybeans than cotton (Luttrell et al. 1982b; Forschler et al. 1992).

In order to determine the efficacy of *Hear*NPV as a biopesticide, several studies were conducted to analyze the effects of nozzle arrangement, as well as the mortality and defoliation depending on larval instar. In most cases, defoliation was determined by frass production. Flusche et al. (1986) found that larvae infected with the virus produce comparable amounts of frass as the uninfected larvae up to the stage in which death occurs; however, as viral dose is increased, frass production decreases. The last larval stage accounts for 81% of the total larval frass, while the 1^{st}, 2^{nd}, and 3^{rd} account for 0.2, 0.9, and 4% respectively, which corresponds with the amount of damage that is done by these instars. Stacey et al. (1977a) found early instar larvae damaged 4.4 squares and 1.6 bolls per larva, and damage was reduced by approximately 50%

when 1st-3rd instar larvae were released after an application of *Hear*NPV. Stacey et al. (1977c) found the mortality of 1st-3rd instar larvae was greatest when the fruit and lower canopy were treated, and that nozzle arrangement and orientation did not influence efficacy, which was consistent with Stacey et al. (1979). Alam et al. (1987) found infected 1st, 2nd, and 3rd instars reduced feeding and frass development when infected, and Luttrell et al. (1982a) found the mortality of larval instar decreased as the initial instar at the application of *Hear*NPV increased. Ignoffo et al. (1978) determined *Hear*NPV resulted in high levels of control with little crop damage when the population was still in the early instars and high doses of the virus were used. Therefore, if inoculation occurs at early instars death can occur before the larvae reach the last and most damaging instar, and efficacy of *Hear*NPV is not determined by nozzle arrangement or orientation, but is determined by coverage and host larval instar. *Hear*NPV seems to be an effective biopesticide on earlier instar larvae, but ultraviolet radiation and other environmental factors should be considered when applying this virus.

Vertical Transmission:

Vertical transmission results from the larvae being exposed to the virus at a later instar and surviving through pupation to adulthood. Most Baculoviridae, including *Hear*NPV, that infect the adult host can be transferred from the adult to its progeny through transovum transmission (Hamm and Young 1974; Andreadis 1987; Zhou et al. 2005). Studies have been conducted on the vertical transmission of *Hear*NPV, concluding a potential for surviving larvae to have slower development rates, reduced reproductive capacity, and lower weights (Luttrell et al. 1982a; Rothman and Myers 1996; Kukan 1999; Zhou et al. 2005). The reduction in fecundity can be as high as 22% (Rothman and Myers 1996). When a female moth that developed from an

infected larva mates with a male, infected or not, and begins ovipositing, the resulting larvae had a viral mortality of 17-32% as compared with the untreated larvae which had a 3.6% viral mortality (Zhou et al. 2005). However, when the female was healthy and the male was inoculated the viral mortality did not differ from the untreated larvae. Vertical transmission occurs at much lower rates than horizontal transmission, but it might be important in the natural persistence of latent virus in the environment (Kukan 1999; Zhou et al. 2005).

Horizontal Transmission:

Horizontal transmission occurs when virus from infected larvae is transmitted to uninfected larvae (Andreadis 1987). This mode of transmission is the major factor for the development and continuation of epizootics (Fuxa 1989). Horizontal transmission of *Hear*NPV has several well-documented transmission paths: abiotic, larva-to-larva, parasitoid-to-larva, and predator-to-larva.

Abiotic Transmission

Varying concentrations of viral occlusion bodies in soil can, to varying degrees and under the right conditions, be transported to the understory of plants and be taken up by *H. zea*, inducing an epizootic event (Fuxa and Richter 2006). In an experiment conducted by Fuxa and Richter (2006), it was found that under ideal precipitation conditions the number of occlusion bodies on the plant increased as the viral concentration of soil increased; however, as they moved from the lower 7.87in of the plant there were significantly fewer occlusion bodies. It was also found that mortality decreased as the height increased for each soil concentration level. Fuxa and Richter (2006) also looked at wind as a mode of transmission from soil to plant. More occlusion

bodies made it to the plant when the concentration was higher in the soil, and occlusion body concentration on the plant decreased as plant height increased. Fuxa and Richter (2001) found that the amount of occlusion bodies transported from the soil to plants did not differ between 0.2 and 2in of precipitation, and no virus was transmitted where there was no rain. Young (1990) determined that rainfall of 0.01in was enough to disperse the virus in soybeans, and could be an important means of secondary transmission. The wind as a mode of transmission was able to initiate infection rates between 0.5-31% in first instar *H. virescens* (Fuxa and Richter 2001). All experiments showed that some physical force was required to transport the viral occlusion bodies from the soil to the plant, and that soil composition, wind, and precipitation all play a role in transmission rates.

Viral occlusion bodies can penetrate deep into the soil profile, but once there they are ineffective in creating or sustaining an epizootic event. Young and Yearian (1986) showed that the viral activity decreases during the summer, but remains viable. Also, they determined that tillage can increase the level of activity in the upper layer of soil, which is supported by Fuxa and Richter (1995) who found no reduction in viral occlusion bodies following tillage or other cultural practices. Young and Yearian (1986) determined that as viral concentration applied to the soil increased, a higher number of occlusion bodies were active on the plant. Several studies have been conducted on the longevity of viral occlusion bodies in the soil, as well as threshold concentrations and depths (Fuxa et al. 2001; Fuxa et al. 2007; Fuxa 2008). Fuxa (2008) found that soil concentration thresholds ranged from 0.88 to 81.5 OB/oz of soil to result in a 2% mortality rate, which was assumed to be high enough to induce an epizootic event.

Larval Vectors

The most obvious form of transmission is from infected larva to uninfected larvae (Ali et al. 1985; Flusche 1986; Ali et al. 1987a; Ali et al. 1987b; Vasconcelos 1996). Frass produced from an infected larva can contain *Hear*NPV concentrations high enough to infect healthy larvae that ingest it (Ali et al. 1987a; Vasconcelos 1996). Larvae coated in *Hear*NPV have the potential to spread the virus by depositing it through movement as shown in a study conducted by Ali et al. (1987a). Ali et al. (1985) found that infected larvae released into a healthy population of *H. zea* simulated 5 or 25% mortality levels. Virus transmission occurred between the infected and healthy population, as well as to the second healthy population that was added after all the larvae pupated or died from the first population. This exemplifies the ability for the virus to remain active across populations. Another route of larvae-to-larvae transmission is cannibalism. However, Vasconcelos (1996) determined *H. zea* larvae choose diet over cannibalism more frequently, and when cannibalism is chosen there is no preference for either infected or uninfected larvae. Transmission between larvae of uniform age increased as the number of larvae that were initially infected increased (Ali et al. 1987b). Larvae infected and dying at earlier instars excrete smaller quantities of the virus upon death than larvae in later instars (Ali et al. 1987a). Contamination of feces occurs in infected larvae 3 days after infection; however, surface contamination can result in earlier fecal contamination (Ali et al. 1987a). Surface contamination can also occur by the wounding or liquefaction of the larvae at the later stages of the virus. Secondary transmission, in which healthy larvae in a location that had previously had infected larvae receive an infection, ranged from 6 to 23% mortality in a study conducted by Ali et al. (1985). *Helicoverpa zea* larvae regurgitate as a defensive mechanism, and regurgitation from infected larvae has been found to be a viable mode of transmission (Vasconcelos 1996).

Predator Vectors

Predators are capable of spreading NPVs without being susceptible to them (Young and Hamm 1985; Li et al. 1999). In a study conducted by Young and Yearian (1990b), spiders and *Nabis* spp. most often tested positive for carrying *Hear*NPV; however, *Geocris* spp., *Orius* spp., coccinellids, and reduviid species were also found to be contaminated with *Hear*NPV. Detectable levels of NPV can be present in the excretions of predators that feed on infected larvae 24 hours after feeding until up to 15 days after feeding, depending on the virus and predator (Smirnoff 1959; Capinera and Barbosa 1975; Beekman 1980; Cooper 1981; Abbas and Boucias 1984; Young and Hamm 1985; Young and Yearian 1987; Boucias et al. 1987; Kring et al. 1988; Li et al. 1999; Lee and Fuxa 2000a, 2000b). Predators also have the potential to vector *Hear*NPV by feeding on infected larvae and transmitting the virus to healthy larvae that the predator feeds on thereafter. Young and Yearian (1990b) conducted a field study looking at the potential for predators within soybeans to vector *Hear*NPV. They found a complex of predators: spiders; *Geocoris* spp.; *Orius* spp.; coccinellid larvae; *Nabis* spp.; and reduviid species were contaminated; however, they did not appear to be important in the overall viral dissemination.

Parasitoid Vectors

Parasitoids, such as *Apanteles glomeratus* (Hymenoptera: Braconidae) and *Microplitis croceipes*, can become infected by emerging from an infected host or contaminated by an application (Raimo et al. 1977; Levin et al. 1983; Young and Yearian 1989; Young and Yearian 1990a). Parasitoids contaminated by application can transmit the virus mechanically to uninfected hosts. Parasitoids that emerge from infected hosts can transmit the virus by

oviposition, and uninfected parasitoids that oviposit into infected hosts have the potential to subsequently transmit the virus through a contaminated ovipositor (Raimo et al. 1977; Young and Yearian 1989; Young and Yearian 1990a). Young and Yearian (1989) showed that *M. croceipes* that emerge from infected larvae can transmit *Hear*NPV; however, the role in disseminating the virus is likely to be small. Young and Yearian (1990a) determined that *M. croceipes* coated in the virus were a much better vector than a parasite that only had a contaminated ovipositor. They also determined *M. croceipes* that were previously exposed to infected larvae were able to transmit the virus to uninfected larvae when the parasitoid population was at a density of four parasitoids per meter of row. *M. croceipes* with painted ovipositors did not transmit the disease, and *M. croceipes* that had been completely painted with the virus were the best at transmitting the virus. As with the density of infected larvae, the more infected parasitoids there were per unit area the higher mortality was. *Apanteles glomeratus* parasitoids that emerged from infected *Pieris rapae* (Lepidoptera: Pieridae) larvae were also capable of transmitting a granulovirus (Baculoviridae) (Levin et al. 1983). Young and Yearian (1989) found 13% of females that emerged from infected larvae transmitted the virus, with a mean mortality of 20%. However, direct mortality from parasitoids emerging from infected larvae in the field appeared to be between 2.6 and 4.9%. McCutchen et al. (1996) found that although NPVs did not directly affect the parasitoids, there appeared to be an increase in their rate of development, resulting in a smaller size.

Knowledge Gaps and Objectives

There have been no studies performed to understand how quickly *Hear*NPV spreads across a soybean field. Also, there does not appear to be adequate information on the potential

feeding of arthropods on infected larvae at the liquefaction stage and their potential for carrying the virus. In addition, while there have been some studies done testing parasitoids and predators coated in the virus which proved to be methods of transmission, studies have failed to analyze whole field species complexes as potential carriers upon the application of *Hear*NPV. Finally, there appears to be a lack of studies analyzing the mode of transmission from predators or off-target arthropods or parasitoids to uninfected larvae. No study has been conducted to determine the time *Hear*NPV is active in the canopy of a soybean field after an application. The current study was conducted in order to better understand the potential transmission of each vector. The first objective was to determine how long *Hear*NPV was active in the crop canopy and how far it disseminated. Then, identify the major obligate carriers of *Hear*NPV. The next objective was to determine the identity, frequency, and importance of facultative carriers of *Hear*NPV in a soybean field infested with infected *H. zea* larvae. The final objective was to determine the efficacy of *Hear*NPV through time to mortality, percent defoliation, and potential to transmit to a second generation. Each of these objectives will help in determining the efficacy of *Hear*NPV by better understanding the potential for horizontal transmission.

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CHAPTER TWO: Horizontal transmission of *Helicoverpa armigera* Nucleopolyhedrovirus in soybean fields infested with *Helicoverpa zea*

Abstract:

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) is a viral biopesticide in the family Baculoviridae, which are known for their ability to induce epizootic events. Horizontal transmission occurs through several pathways involving abiotic factors, such as soil, wind, and rain, and biotic factors such as predators, parasitoids, and infected hosts. Understanding horizontal transmission of *Hear*NPV is important in understanding the efficacy of *Hear*NPV as a bio-insecticide. This study was conducted to determine the ability of *Hear*NPV to disseminate across a soybean field, determine the time *Hear*NPV is active in the crop canopy, and determine the importance of non-host arthropods present during the application in the dissemination of *Hear*NPV. This was determined by making an application of *Hear*NPV in a 50 x 50 foot area, with sample distances of 0, 0-25, 25-50, 50-100, and 100-200 feet from the application area. Each area was sampled 3, 7, 10, 14, and 21 days after the application.

In 2016, *Hear*NPV disseminated 100 feet by 7 days, and remained in the canopy for 14 days. In 2017, *Hear*NPV disseminated 200 feet in all three fields, and remained in the canopy for 13-21 days. Three-cornered alfalfa hoppers and *H. zea* larvae were most commonly determined to be carriers of *Hear*NPV, which was capable of disseminating 200 feet within 3 days, and when a sustained population of *H. zea* was present *Hear*NPV remained active in the canopy. Certain arthropods, mainly the host, three-cornered alfalfa hoppers, and big-eyed bugs, were associated with the presence of *Hear*NPV, while spiders and grasshoppers (Acrididae and Tettigoniidae) appeared to be associated with its absence.

Introduction:

Helicoverpa zea (Boddie), is considered the most damaging pest of soybean (*Glycine max* (L.) Merrill) throughout the Mid-South United States (Musser et al 2015a). In soybean, *H. zea* is capable of causing both defoliation and fruit damage, resulting in yield loss (Mueller and Engroff 1980; Adams et al. 2015). Once a field is infested with *H. zea* larvae, natural enemies can suppress the growing population, however, once the economic threshold is reached an insecticidal application is necessary (Cabanillas and Raulston 1996; Sansone and Smith 2001; Pfannenstiel and Yeargan 2002). *Helicoverpa zea* have become resistant to several insecticidal classes such as chlorinated hydrocarbons, organophosphates, and organochlorines, and are developing resistance to the commonly used pyrethroid class (Wolfenbarger et al. 1971; Sparks 1981; Abd-Elghafar et al. 1993; Kanga et al 1996; Musser et al. 2015b). Other avenues for controlling *H. zea* larvae are being explored, such as the development of new insecticide classes and research on potential biocontrol agents. One biocontrol agent that is being studied, and is used extensively in other countries for the control of Heliothines, is *Helicoverpa armigera* Nucleopolyhedrovirus (*Hear*NPV).

*Hear*NPV is a viral biocontrol agent that is used to control *H. zea* populations with no known off-target effects (Young and McNew 1994). It is in the viral family Baculoviridae, whose members are known for their proteinaceous occlusion bodies that protect the viral DNA from extended exposure to the environment (Bilimoria 1986; Bilimoria 1991). An infected larva will eventually liquefy and release occlusion bodies into the environment where the virus will repeat its life cycle through horizontal transmission (Boucias and Pendland 1998).

Horizontal transmission can occur through several routes. Abiotic conditions such as rainfall and wind can transport occlusion bodies from the soil to the crop canopy where infection

can occur (Young 1990; Fuxa and Richter 2001; Fuxa and Richter 2006). Infected larvae are capable of transmitting *Hear*NPV through cannibalism by a healthy larva (Vasconcelos 1996). Infected larvae can defecate viral particles in adequate concentrations to initiate infection when consumed, as well as spread *Hear*NPV through surface contamination by vomiting, liquefaction, or movement (Ali et al. 1987a; Ali et al. 1987b; Vasconcelos 1996). Parasitoids, such as *Microplitis croceipes* (Hymenoptera: Braconidae), transmit *Hear*NPV when they emerge from infected larvae and oviposit into healthy larvae, or when they contaminate their ovipositor by laying an egg in an infected larva (Young and Yearian 1989; Young and Yearian 1990a). Predators such as *Nabis* spp. (Hemiptera: Nabidae), *Reduviid* spp. (Hemiptera: Reduviidae), *Geocoris* spp. (Hemiptera: Geocoridae), *Orius* spp. (Hemiptera: Anthocoridae), coccinellid larvae, and spiders (Araneae) can act as carriers of *Hear*NPV (Young and Yearian 1990b; Lee and Fuxa 2000a; Lee and Fuxa 2000b). *Nabis roseipennis* can feed on an infected larva and then defecate frass that contains a high enough viral concentration to cause infection when ingested up to 10 days after feeding (Young and Yearian 1987). A more effective application of *Hear*NPV could potentially be made in which an epizootic has a greater probability of occurring if the role of the arthropod complex present in a soybean field in viral dissemination were better understood. Also, understanding how quickly *Hear*NPV disseminates and how long it is active in the crop canopy could lead to *Hear*NPV being more readily adopted as a potential for control of *H. zea* among growers.

The first objective of this study was to determine the ability of *Hear*NPV to disseminate across a soybean field. The second objective of this study was to determine how long *Hear*NPV would remain present in the crop canopy. The third objective of this study was to ascertain the importance of arthropods present within the field during the application in the dissemination of

*Hear*NPV. The initial hypothesis was that *Hear*NPV was capable of spreading outside the application area, and would remain in the crop canopy. All arthropods which encountered the application were considered to be possible carriers of *Hear*NPV.

Materials and Methods:

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) was released in soybean at four sites near Lonoke, AR. The virus was released in an application area (50 by 50 feet) located on the edge of the fields. The remaining portions of each field was left untreated, and sampling zones were established at 0-25, 25-50, 50-100, and 100-200 feet from the application area, before application of the virus was initiated. *Hear*NPV was obtained from AgBiTech (Heligen, AgBiTech Corporation, Queensland, Australia) at a viral concentration of $2.22x10^{11}$ occlusion bodies/ fl. oz. The virus was applied at a rate of 1.6 fl. oz/acre using a CO_2 backpack sprayer applying a spray volume of 10 gal/acre. The first of the four application areas was treated on 5 August, 2016, two application areas were treated on 7 August, 2017, and one application area was on 21 July, 2017.

All samples were collected with a standard 15 inch sweep net (BioQuip Products, Rancho Dominques, CA). When samples were taken, at least one step was taken between sweeps to insure the same area was not swept twice. During a sweep, the sampler focused on a single row; however, it was common for the two adjacent rows to be sampled as well due to the average sweep length being between four and five feet. For each sweep, the top of the sweep net contacted the crop under the canopy approximately mid-way up the stem to ensure arthropods present would fall into the net. Three samples consisting of 10 sweeps each were taken prior to the application to verify that no natural infestation of *Hear*NPV was present in each field. In

2016, arthropod samples were taken 3, 7, 14, and 21 days after the application. Three samples, each consisting of 10 sweeps, were taken for each distance, including the application area, for each sample date. In 2017, the two fields sprayed on 7 August were sampled 2, 6, 9, 13, and 20 days after the application, and the field sprayed on 21 July was sampled 3, 7, 10, 14, and 21 days after the application. Each sample date consisted of 3 samples from the application area and 3 from the 0-25' area at 10 sweeps, 3 samples from the 25-50' area at 12 sweeps, 5 samples from the 50-100' area at 21 sweeps, and 6 samples from the 100-200' area at 58 sweeps, for a total of 20 samples per sample date. This increase in sample size allowed for equivalent proportions of each area to be sampled. All samples were frozen in a 4°C freezer for a minimum of 48 hours to ensure arthropod mortality.

Throughout the experiment, precautions were taken to minimize anthropogenic movement. During the application, the applicator did not leave the application area until the plot was sprayed, and then walked directly out of the field. Only the applicator and samplers traveled through the trial area, and samplers took samples from the farthest distance first and then moved towards the application area. All samples for one distance were taken sequentially, and then the sampler acquired a new sweep net and moved to the adjacent sampling area.

Arthropods present in samples were identified and counted before being placed into a 15ml test tube and homogenized. In 2017 the first sample date was divided into five subsamples: the four most abundant arthropods individually, and all remaining arthropods in a single pooled sample. Occlusion bodies were extracted from the emulsified sample using a modified extraction technique (O'Reilly et al. 1992), and stored in a 4°C freezer. Viral DNA was extracted from the occlusion bodies using a DNA extraction kit, DNeasy Blood and Tissue Kit (Quiagen, Hilden, Germany), and stored in a -20°C freezer. Following viral DNA extraction, polymerase chain

reaction (PCR) was used to replicate any viral DNA present using HearNPV specific primers (IDT, Coralville, IA), and a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). A known positive sample and a known negative sample were also added to the thermocycler before each run of PCR to confirm the amplification process was successful. After the amplification of the DNA by PCR, samples were processed using gel electrophoresis, loading 20µL of each sample PCR product into individual wells. The gel was run for 1 hour at 90 volts using Sybr Safe DNA gel stain (Life Technologies Corporation, Carlsbad, CA), and was visualized under an ultraviolet base-light (UPV LLC., Upland, CA). If a band was present at 450 base pairs, HearNPV was considered positive for that corresponding sample. Main effects consisted of presence of each arthropod, number of each arthropod, presence of *Hear*NPV, and interaction effects between presence of each arthropod and presence of *Hear*NPV, and number of each arthropod and presence of *Hear*NPV. Also, sample distance, sample date, and presence of *Hear*NPV by sample date or sample distance were analyzed as main effects. Site year, and field location were analyzed as random effects. These were then analyzed using an ANOVA (α=0.05), followed by Fisher's Exact Test in order to determine independence, due to the response variable being categorical in nature. The mean number of *H. zea* larvae was analyzed using an ANOVA (α=0.05) and Tukey's post hoc. SAS 9.4 was used for all data analysis (PROC GLIMMIX. Version 9.4, SAS Institute Inc., Cary, NC).

Results:

In 2016 *Hear*NPV was observed 100 feet from the application area by 7 days after the application, and remained in the crop canopy 14 days (Table 1). In the first field sprayed in 2017, virus spread 200 feet 2 days after the application, and was present in the crop canopy for 13 days.

The second field sprayed in 2017 showed the same initial rate of spread as the first field in 2017, but virus was active in the crop canopy 20 days after the application. Positive samples of the virus were observed as far as 200 feet for each sample date, including 21 days after initial application for the third field sprayed in 2017 (Table 1).

Helicoverpa zea populations differed across fields and sample dates. In 2016 Field 1, *H. zea* populations were well above the average action threshold of 8 larvae per 25 sweeps for both the 3 and 7 days after application sample dates, significantly higher than all other fields, before populations dropped to 2.3 larvae per 25 sweeps, well below the action threshold (Table 1). For 2017 Field 1, populations never reached threshold, but dropped from 5.6 larvae per 25 sweeps at 3 days, which was no different than any other field sprayed in 2017, to 0.4 larvae at 21 days. In 2017 Field 2, threshold was also never reached, and populations dropped from 6.9 larvae 3 days after to 0.6 larvae 21 days after application. In 2017 Field 3, *H. zea* populations never went below the threshold, staying between 9 and 14 larvae per 25 sweeps through 21 days after application. This field had significantly higher populations than all fields 10, 14, and 21 days after application, and significantly higher populations than the other 2017 fields 7 days after application (Table 1).

When the population size of *H. zea* larvae was analyzed with *Hear*NPV presence by field using Fisher's Exact Test, no association was discovered except for Indian Bayou South, which had a positive association. An increase in *H. zea* larval populations was associated with presence of *Hear*NPV. However, when presence, not abundance, of *H. zea* larvae was analyzed with *Hear*NPV presence using Fisher's Exact Test, a positive association was discovered across all fields.

The average arthropod complex found in a soybean field was determined by inventorying each sample from all fields. Across all fields, *H. zea* larvae was the most commonly found species of arthropod (Table 2). Three-cornered alfalfa hoppers (*Ceresa festina* (Hemiptera: Membracidae)) were the second most abundant species, and grape colaspis (*Colaspis brunnea* (Coleoptera: Chrysomelidae)) adults were third. Green cloverworms (*Hypena scabra* (Lepidoptera: Erebidae) and Diptera (Sarcophagidae, Calliphoridae, Tachinidae, Muscidae, and Tephritidae) were the fourth and fifth most common arthropods detected. Spiders (Araneae) were the sixth, while big-eyed bugs (*Geocoris* spp. (Hemiptera: Geocoridae)) and tarnished plant bugs (*Lygus lineolaris* (Hemiptera: Miridae)) were seventh and eighth respectively (Table 2).

In 2017, three-cornered alfalfa hoppers were positive for *Hear*NPV in 21 out of 58 subsamples ranging from 0 to 200 feet (Table 3). This was more than any other arthropod present. *Helicoverpa zea* and big-eyed bugs were positive in 13 out of 58, and 12 out of 26 subsamples respectively, with infected *H. zea* larvae present in all distances while big-eyed bugs were present in all but the application area. Spiders were positive for *Hear*NPV in 9 out of 26 subsamples across 3 distances: 25, 100, and 200 feet (Table 3). These 4 arthropods account for 74% of the positive subsamples. The other 26% was comprised of spotted cucumber beetles (*Diabrotica undecimpunctata* (Coleoptera:Chrysomelidae)) in 4 out of 13 subsamples, yellow-striped armyworms (*Spodoptera ornithogalli* (Lepidoptera: Noctuidae)) in 4 out of 6 subsamples, green cloverworms in 5 out of 14 subsamples, grape colaspis in 2 out of 2 subsamples, bean leaf beetles (*Cerotoma trifurcate* (Coleoptera: Chrysomelidae)) in 2 out of 6 subsamples, and grasshoppers (Orthoptera: Acrididae and Tettigoniidae) in 2 out of 9 subsamples (Table 3).

Discussion:

According to Tanada and Fuxa (1989), key factors in inducing an epizootic are categorized under two broad terms: host population and pathogen population. The pathogen population is further divided by the pathogen's ability to disperse, which is determined by physical conditions, host, and carrier factors. Both biological factors are based on behavior and movement. In this study physical conditions were assumed static across replications, and pathogen populations were controlled by using a constant application area and rate. *Helicoverpa zea* was the only biological host of *Hear*NPV present in any field, and the arthropod complex was identified by inventorying all samples. An association for larger populations of *H. zea* and *Hear*NPV presence could not be determined for all fields, however presence of *H. zea* was associated with presence of *Hear*NPV. In the 2017 Field 3, a sustained population of *H. zea* was present, with averages never dropping below 9 larvae per 25 sweeps. This was the only field where a sustained population occurred. This field also had the most *Hear*NPV-positive samples, and the most sustained dissemination. This could imply that a sustained population of *H. zea* larvae are essential for the continuation of an epizootic event. In this circumstance *Hear*NPV could be an invaluable product, capable of controlling several generations of *H. zea*. Future studies should focus on the importance of *H. zea* larvae in sustaining *Hear*NPV dissemination by controlling for population sizes of *H. zea* larvae.

Some carriers of *Hear*NPV have been studied extensively by Young and Yearian (1989; 1990a; 1990b), who identified *Nabis* spp., *Reduviid* spp., *Geocoris* spp., *Orius* spp., coccinellid larvae, and spiders as carriers. However, their significance in inducing an epizootic was determined to be minimal. This study found several arthropods as potential carriers through

contamination via overspray. Three-cornered alfalfa hoppers were the most numerous arthropod collected across the fields, and were the most commonly confirmed arthropod carrying *Hear*NPV. Big-eyed bugs were found to be carriers almost as often as the host *H. zea* larvae. Three-cornered alfalfa hopper, big-eyed bugs, *H. zea* larvae, and spiders were responsible for 74% of the virus positive subsamples, indicating them as a primary source of viral dissemination and potential induction of an epizootic event. This was further supported for three-cornered alfalfa hoppers, big-eyed bugs, and *H. zea* larvae through Fisher's Exact Test, which showed positive viral samples were not independent of these populations ($p<0.001$), and that when these arthropods are present there is an association for *Hear*NPV being detected. The presence of *Hear*NPV was also shown to not be independent of spiders, but when spiders were present there was an association for *Hear*NPV to not be detected. This suggests that spiders prey on potential carriers of *Hear*NPV, and act as dead-end hosts. Several arthropods such as three-cornered alfalfa hoppers, spotted cucumber beetles, yellow-striped armyworms, green cloverworms, grape colaspis, bean leaf beetles, and grasshoppers are reported here as carriers of *Hear*NPV for the first time. These previously unknown carriers could be an important piece in understanding horizontal transmission of *Hear*NPV in a soybean field.

*Hear*NPV can spread 0-200 feet as quickly as 3 days, and can remain viable in the crop canopy for at least 21 days under certain environmental and ecological conditions. Presence of *H. zea,* three-cornered alfalfa hoppers, and big-eyed bugs were associated with *Hear*NPV presence; when these arthropods were present *Hear*NPV was more likely to remain in the crop canopy for an extended period of time. Presence of spiders was negatively correlated with presence of *Hear*NPV, possibly resulting from stationary spiders predating on disseminating arthropods or find arthropods carrying *Hear*NPV unpalatable. *Hear*NPV appears to be a viable

bio-insecticide, capable of disseminating up to 200 feet and remaining in the field for 21 days when proper conditions are present; however, future studies should be conducted to better understand the potential each arthropod identified as a carrier has in horizontal transmission.

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Table 2.1: *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larval populations evaluated at 25 sweeps, and maximum observed distance *Helicoverpa armigera* Nucleopolyhedrovirus spread for all fields and sample dates where *Hear*NPV was applied.

Date	Days After App.	Mean # *H. zea* / 25 sweeps	Max Distance (ft)
		2016 Field 1	
8 Aug. 2016	3	36.7 a, A	0
12 Aug. 2016	7	24.3 b, A	100
19 Aug. 2016	14	2.3 c, B	100
26 Aug. 2016	21	2.3 c, B	-
		2017 Field 1	
9 June 2017	2	5.6 a, B	200
13 June 2017	6	2 b, C	0
16 June 2017	9	1.7 b, B	100
20 June 2017	13	1.1 b, B	100
27 June 2017	20	0.4 b, C	-
		2017 Field 2	
9 June 2017	2	6.9 a, B	200
13 June 2017	6	1.7 b, C	-
16 June 2017	9	0.5 b, B	-
20 June 2017	13	0.3 b, B	-
27 June 2017	20	0.6 b, C	200
		2017 Field 3	
24 July 2017	3	9.8 a, B	200
28 July 2017	7	13.9 a, B	200
31 July 2017	10	12.8 a, A	200
4 Aug. 2017	14	9 a, A	200
11 Aug. 2017	21	9.3 a, A	200

[1]Lowercased letters denote a significantly different value within a given field using an ANOVA (α=0.05), and a Tukey's post hoc analysis ($p<0.05$).
[2]Capital letters denote a significantly different value across fields for a given sample date using an ANOVA (α=0.05), and a Tukey's post hoc analysis ($p<0.05$).

Table 2.2: The ten most prevalent arthropods captured and identified in all samples collected from all sample dates across all four fields where *Helicoverpa armigera* Nucleopolyhedrovirus was applied, with the numbers for each arthropod at each field and total numbers across fields given.

	H. zea[1]	TCAH[2]	GC[3]	GCW[4]	FLY[5]	SPIDERS	BEB[6]	TPB[7]	SCB[8]	GH[9]
2016 Field 1	394	194	29	319	325	108	47	16	66	42
2017 Field 1	218	784	423	62	46	143	111	129	14	24
2017 Field 2	180	675	537	70	45	224	151	54	18	8
2017 Field 3	1028	108	21	232	90	21	14	2	33	14
Totals:	1820	1761	1010	683	506	496	323	201	131	88
Ranks:	1	2	3	4	5	6	7	8	9	10

[1]*Helicoverpa zea* larvae, [2]Three-cornered alfalfa hopper, [3]Grape colaspis, [4]Green cloverworm, [5]Diptera (Sarcophagidae, Calliphoridae, Tachinidae, Muscidae, and Tephritidae), [6]Big-eyed bugs, [7]Tarnished plant bugs, [8]Spotted Cucumber Beetle, [9]Grasshopper (Acrididae and Tettigoniidae).

Table 2.3: All arthropods confirmed as carriers of *Helicoverpa armigera* Nucleopolyhedrovirus through PCR from the first sample date, their distribution and frequency for all sampled areas, as well as the total number of positive samples for each carrier across all fields where *Hear*NPV was applied.

Distances (ft)	TCAH[1]*	*H. zea*[2]*	BEB[3]*	SPIDERS[4]*	SCB[5]	YSAW[6]	GCW[7]	GC[8]	BLB[9]	GH[10]
0	4	5	0	0	1	2	4	0	0	0
25	3	1	1	2	0	0	0	0	1	0
50	2	2	2	0	0	0	0	0	0	0
100	5	1	4	3	0	1	1	0	0	1
200	7	4	5	4	3	1	0	2	1	1
Total	21	13	12	9	4	4	5	2	2	2

[1]Three-cornered alfalfa hopper (n=44), [2]*Helicoverpa zea* larvae (n=58), [3]Big-eyed bug (n=26), [4]Spiders (n=26) [5]Spotted cucumber beetle (n=13), [6]Yellow-striped armyworm (n=10), [7]Green coverworm (n=23), [8]Grape colaspis (n=2), [9]Bean leaf beetle (n=6), [10]Grasshoppers (Acrididae and Tettigoniidae) (n=10).

***** Denotes association ($p<0.05$) with positive PCR samples by using Fisher's Exact Test ($\alpha=0.05$).

CHAPTER THREE: The importance of arthropods in the dissemination of *Helicoverpa armigera* Nucleopolyhedrovirus in a soybean field

Abstract:

The movement of *Helicoverpa armigera* Nucleopolyhedrovirus (*Hear*NPV) in a field setting is not well understood, however, successful modes of horizontal transmission have been extensively researched. Studies examining horizontal transmission through biological carriers concluded they were likely not significant in increasing infection rates. Movement of *Hear*NPV beyond 200ft has been observed in soybean fields infested with *Helicoverpa zea*, but it is not clear which factors are inducing these epizootic events. This study attempts to identify important carriers of *Hear*NPV that acquire the virus through contact with a virus-infected larva. A 50 x 50 foot area was infested with late-stage HearNPV-infected larvae and sample zones of 0, 0-25, 25-50, 50-100, and 100-200 feet from the infestation area were monitored. These zones were sampled 3, 7, 10, 14, 17, and 21 days after the infestation, and analyzed using PCR for viral presence. Species in contact with virus-infected larvae were also confirmed through observation and sentinel game cameras.

The largest amount of viral movement was 3 days after infestation, with *Hear*NPV being detected up to 200 feet away. After 3 days, only one field contained positive samples, which is likely due to only low levels of *H. zea* being present. Several previously undocumented arthropods were identified as carriers of *Hear*NPV, including several families within Diptera, Hemiptera, and others. Results from this study indicate that many previously undocumented arthropod sources of horizontal transmission are likely crucial in spreading *Hear*NPV, as arthropods carrying *Hear*NPV were found up to 200ft away from infected larvae.

Introduction:

Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae), is a major pest in several crops, and is the most damaging pest of soybean (*Glycine max* (L.) Merrill) in the Mid-South United States (Musser et al. 2015a). Once *H. zea* populations within a field reach the economic threshold, an insecticidal application is warranted. However, *H. zea* has developed resistance to many insecticide classes once commonly used for control, including chlorinated hydrocarbons, organophosphates, and organochlorides (Brazzel 1963; Graves et al. 1963; Lingren and Bryan 1965; Adkisson and Nemec 1967; Carter and Phillips 1968; Plapp 1971; Wolfenbarger et al. 1971; Lentz et al. 1974; Sparks 1981;). *H. zea* have also recently developed resistance to pyrethroids (Abd-Elghafar et al. 1993; Kanga et al. 1996; Musser 2015b). Considering that *H. zea* has an affinity for developing resistance, there is a dire need for new insecticide chemical classes, especially those that are effective, economical, and not excessively harmful to humans.

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) is a virus in the family Baculoviridae that is specific to Heliothines. *Hear*NPV is a large, double stranded DNA virus that is enveloped and occluded (Bilimoria 1991). The occlusion body is a protein crystalline structure that protects viral DNA from quick degradation by environmental factors, and allows for long-term survival of the virus in the soil (Fuxa et al. 2001; Fuxa et al. 2007; Fuxa 2008). *Hear*NPV is commercially available, relatively inexpensive, and has the potential to create an epizootic event through horizontal transmission (Gröner 1986; Fuxa and Tanada 1987; Inceoglu et al. 2006). Horizontal transmission of *Hear*NPV occurs through many different vectors, including abiotic factors, virus-infected larvae, predators, and parasitoids, which can promote epizootic events, increased infection rates, leading to increased control of *H. zea.* However, most studies found biotic factors to have a minimal role in the development of a *Hear*NPV epizootic

event (Ali et al. 1985; Young and Yearian 1989; Young and Yearian 1990a; Young and Yearian 1990b; Vasconcelos 1996; Fuxa and Richter 2006).

Several studies have revealed the potential for arthropods to transmit *Hear*NPV through deposition of contaminated frass; however, no studies have explored the potential of dissemination by non-parasitic or predatory arthropods in contact with infected larval hosts. The first objective of this study was to determine the ability of *Hear*NPV to disseminate from an infected larva when no healthy host was present. It was hypothesized that movement would be extensive, with potential carriers capable of flying long distances. The second objective of this study was to determine how long *Hear*NPV stayed active in the crop canopy with no host to replicate in. It was hypothesized that *Hear*NPV would not be active in the crop canopy more than 10 days, based on research conducted by Young and Yearian (1987). The third objective of this study was to identify arthropods potentially responsible for the dissemination of *Hear*NPV. It was hypothesized several non-predaceous, non-parasitic arthropods would be identified. The fourth objective of this study was to determine the importance of arthropods identified as carriers in disseminating *Hear*NPV. It was hypothesized that arthropods most often observed in contact would be most important in dissemination.

Materials and Methods:

Helicoverpa zea larvae acquired from Benzon Research Inc. (Carlisle, PA) were fed diet contaminated with *Hear*NPV for a minimum of 24 hours and then observed for viral symptoms prior to use. The virus used was acquired from AgBiTech (AgBiTech Corporation, Queensland, Australia), at a concentration of 2.22×10^{11} occlusion bodies/fl. oz. Larvae of *H. zea* inoculated with *Hear*NPV at the third instar were manually placed into the infestation areas (50 by 50 feet)

located on an edge of 3 soybean fields near Lonoke, AR. The remaining portions of each field was left uninfested. Sample distances of 0-25, 25-50, 50-100, and 100-200 feet from the infestation area were then established. Three samples consisting of 10 sweeps were taken before the infestation to verify that no natural infestation of *Hear*NPV was present.

In 2016, one field was infested on 4 August with 90 infected *H. zea* larvae. Three samples consisting of 10 sweeps were taken at each distance including the infestation area. Samples were taken 3, 7, 14, and 21 days after infestation, with samples consisting of 3 sets of 10 sweeps per sampling area. In 2017, two fields were infested, one on 19 June and the other on 19 July, at a rate of 1 larva per 2 row feet. The field infested on 19 June was infested with 283 larvae, and sampled 3, 7, 14, 17, and 21 days after the application. The field infested 19 July was infested with 410 larvae, and sampled 3, 7, 10, 14, and 22 days after the application. Each sample date for the 2017 field season consisted of 3 samples from the application area and 3 from the 0-25' area at 10 sweeps each, 3 samples from the 25-50' area at 12 sweeps each, 5 samples from the 50-100' area at 21 sweeps each, and 6 samples from the 100-200' area at 58 sweeps each, for a total of 20 samples per sample date. This increase in sample size allowed for equivalent proportions of each area to be sampled. All samples from both years were frozen for a minimum of 48 hours to ensure arthropods were killed. Infected larvae within the infestation area were monitored using Bushnell NatureView trail cameras (Bushnell Corporation, Overland Park, KS) and manual observations in order to determine what arthropod species were in contact with them.

Arthropods present in samples were identified and quantified before being placed into a 15mL test tube and homogenized. In 2017 only, the first sample date was divided into five subsamples: the four arthropods most commonly observed in contact with infected larvae and all

other arthropods. The occlusion bodies were extracted from the homogenized sample using a modified extraction technique (O'Reilly et al. 1992), and stored in a 4°C freezer. The viral DNA was extracted from the occlusion bodies with a DNA extraction kit, DNeasy Blood and Tissue Kit (Quiagen, Hilden, Germany), and stored in a -20°C freezer. Following viral DNA extraction, polymerase chain reaction (PCR) was used to replicate any viral DNA present using HearNPV-specific primers (IDT, Coralville, IA), and a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). A known positive sample and a known negative sample were also added to the thermocycler before PCR to confirm the success of the amplification process. After the amplification of DNA by PCR, samples were processed using gel electrophoresis, where a 20µL aliquot of each sample was loaded into individual wells. The gel was run for 1 hour at 90 volts using Sybr Safe DNA gel stain (Life Technologies Corporation, Carlsbad, CA), and was then visualized under an ultraviolet baselight (UPV LLC., Upland, CA). If a band was present at 450 base pairs, HearNPV was considered positive for that corresponding sample. Main effects consisted of presence of each arthropod, number of each arthropod, presence of *Hear*NPV, and interaction effects between presence of each arthropod and presence of *Hear*NPV, and number of each arthropod and presence of *Hear*NPV. Site year, and field location were analyzed as random effects. All data were subject to Fisher's Exact Test with an alpha value of 0.05, in order to determine association with a response variable that was categorical (PROC FREQ. Version 9.4, SAS Institute Inc., Cary, NC).

Results:

The 2016 field that was infested with 96 infected larvae did not have a single positive sample, however the two fields infested in 2017 at a rate of one larva per two row feet had

several positive samples extending to the 200 foot sample zone at 3 days after the infestation. The 2017 Field 1 was infested with 283 infected larvae, and had positives in all six of the samples at 200 feet, 2 at 100 feet, 2 at 25 feet, and one in the infestation area (Table 1). The 2017 Field 2 was infested with 410 infected larvae, and by 3 days after the application had one sample positive at 200 feet, 3 at 100 feet, and 2 at 50 feet (Table 2). After 3 days only two samples were positive and both were in 2017 Field 2. One sample was positive from the infestation area 10 days after the infestation, and one was positive at the 25ft distance 14 days after (Table 2).

Several arthropods were manually observed in contact with *Hear*NPV infected larvae (Table 3). Soybean nodule flies (*Rivellia quadrifasciata* Macquart (Diptera: Ulidiidae)) were manually observed, and observed using game cameras to be potential facultative carriers of *Hear*NPV by walking on and feeding on infected larvae for long spans of time (Figure 1; Table 3). Soybean nodule flies were also confirmed as carriers of *Hear*NPV using PCR (Table 4). Mirids such as tarnished plant bugs (*Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae)) were also observed feeding on infected larvae, and was confirmed by game camera photos that revealed tarnished plant bugs engorging themselves on infected larvae before moving off lethargically and eventually returning to engorge again (Table 3; Figure 2; Figure 3). Mirids were also confirmed to be carriers of *Hear*NPV with PCR (Table 4). Big-eyed bugs (*Geocoris* spp. (Hemiptera: Geocoridae)) were manually observed feeding on infected larvae, but only one picture was captured with a big-eyed bug approaching an infected larva (Table 3; Figure 4). Big-eyed bugs were confirmed as carriers of *Hear*NPV through PCR (Table 4). Three-cornered alfalfa hoppers (*Ceresa festina* (Say) (Hemiptera: Membracidae)) were manually observed close to infected larvae, but were never seen feeding on them; however, they were confirmed as carriers of *Hear*NPV through PCR (Table 4; Figure 5). Several *Hear*NPV-infected larvae were

observed to be entirely consumed by ant colonies (Hymenoptera: Formicidae) that went so far as to remove leaf material where the larva liquefied (Table 3; Figure 6; Figure 7; Figure 8). Reduviids (Hemiptera: Reduviidae) and spotted lady beetles (*Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae)) were found crawling and feeding on liquefied larval remains (Table 3; Figure 9; Figure 10). Green stink bugs (*Acrosternum hilare* (Say) (Hemiptera: Pentatomidae)) and green lacewing larvae (Neuroptera: Chrysopidae) were also observed as carriers through feeding on liquefied infected larvae (Figure 11; Figure 12). A common genus of ground beetles (Coleoptera: Carabidae), *Lebia*, was also observed feeding on infected larval remains (Table 3; Figure 13). However, none of these species were confirmed as carriers of *Hear*NPV by PCR. Healthy *H. zea* larvae were observed feeding on *Hear*NPV-infected larvae, and wasps (Hymenoptera: Vespidae) were observed carrying and feeding on infected larval remains, with both being confirmed as carriers of *Hear*NPV through PCR (Figure 14; Figure 15; Table 4). Spotted cucumber beetles (*Diabrotica undecimpunctata* Mannerheim (Coleoptera: Chrysomelidae)) were manually observed feeding on infected larvae and proven to be carriers of *Hear*NPV through PCR, but no pictures were obtained (Table 3; Table 4). Milichiidae species were observed feeding on infected larvae along with the dipteran families Sarcophagidae and Tachinidae (Figure 16; Figure 17; Figure 18). Milichiidae species were not ever identified in the samples, therefore were not able to be positively confirmed as carriers; however, Calliphoridae, Sarcophagidae, and Tephritidae were confirmed as carriers through PCR (Table 4).

In the subsamples, tarnished plant bugs were found to be positive in 6 subsamples in all sample distances except the 50 foot zone (Table 4). Flies (Calliphoridae, Sarcophagidae, and Tephritidae) and wasps (Vespidae and Sphecidae) both had 5 subsamples that were positive for *Hear*NPV in the 200 foot zone. Tarnished plant bugs, flies, wasps, and spiders (Araneae)

together were responsible for 70% of the positive subsamples. The soybean nodule fly was positive in 4 subsamples, and big-eyed bugs, spotted cucumber beetles, and green cloverworms were each responsible for 2 positive subsamples (Table 4). However, only neuropteran (p=0.01) and hymenopteran (p=0.001) presence was able to be associated with presence of *Hear*NPV through Fisher's Exact Test. All other arthropods were shown to have an independent relationship with viral presence.

Discussion:

Although previous studies conducted by Young and Yearian (1989; 1990a; 1990b) identified predators and parasitoids as potential carriers of *Hear*NPV, this study showed several previously unobserved, non-predaceous, non-parasitic insects as potentially important carriers. Field studies indicated that arthropods visiting infected larvae could be an important mode of dissemination of *Hear*NPV. This is supported by two fields infested in 2017, where *Hear*NPV was detected three days after the infestation up to 200 feet away. If a population of healthy *H. zea* larvae had been present within the field these sources of virus might have induced an epizootic event. Unfortunately, no such populations were present in the fields studied, leading to only two positive samples in all sample dates after 3 days after the infestation for all fields. This indicates that while these off-target arthropods could be an important factor in the dissemination of *Hear*NPV when host populations are present, they do not appear to be a good means of viral longevity in the field. When compared to the previous chapter, this mode of transmission seems to be less viable than surface contamination, which supports previous work conducted by Young and Yearian (1990a). However, it is likely that the increase in sustained dissemination could be due to the presence of the viral host, *H. zea* larvae. Without the presence of host larvae, *Hear*NPV was likely not able to replicate in any widespread amount, leading to a sharp decline

in the amount of virus present. This is compared to work from the previous chapter where the virus was capable of replicating and creating significant amounts of occlusion bodies in the host larvae, thereby increasing the quantity of virus present in the field beyond the initial application.

Prior to this study, the only known carriers of *Hear*NPV were spiders, *Geocoris* spp. (Hemiptera: Geocoridae), *Orius* spp. (Hemiptera: Anthocoridae), *Nabis* spp. (Hemiptera: Nabidae), *Reduviid* spp. (Hemiptera: Reduviidae), *Microplitis croceipes* (Hymenoptera: Braconidae), and cocinellid larvae (Coleoptera: Coccinellidae) (Young and Yearian 1990a; 1990b). Through manual observations and the use of motion-sensor cameras several insects were observed feeding and taking advantage of the liquefaction of the host larvae. The most observed insects were flies in several families, including the soybean nodule fly, which was observed feeding on liquefied larvae for several minutes at a time. Several carriers of *Hear*NPV have been reported here that could be important in the dissemination of *Hear*NPV and the induction of an epizootic event in the presence of a host population. This study indicates that arthropods important in disseminating the virus include more than predators and parasitoids, but a wide variety of arthropods present in the soybean ecosystem. Future studies should be conducted to better understand the importance of these previously un-observed potential carriers in disseminating *Hear*NPV by utilizing fields containing a population of *H. zea* larvae.

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Table 3.1: *Helicoverpa armigera* Nucleopolyhedrovirus viral presence across all distances sampled as determined through PCR three days after infestation of 2017 Field 1, infested with 283 infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae on 19 June 2017.

	Sample Number					
Distance (ft)	1	2	3	4	5	6
0	P	-	-			
25	-	P	P			
50	-	-	-			
100	-	-	-	P	P	
200	P	P	P	P	P	P

P denotes presence of *Hear*NPV detected for a given sample through PCR.

Table 3.2: *Helicoverpa armigera* Nucleopolyhedrovirus viral presence across all distances sampled as determined through PCR three, ten, and fourteen days after infestation of 2017 Field 2, infested with 410 infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae on 19 July 2017.

Days After Application	Distance (ft)	Sample Number					
		1	2	3	4	5	6
3 DAA	0	-	-	-			
	25	-	-	-			
	50	P	-	P			
	100	P	-	P	P	P	
	200	-	-	P	-	-	-
10 DAA	0	P	-	-			
	25	-	-	-			
	50	-	-	-			
	100	-	-	-	-	-	
	200	-	-	-	-	-	-
14 DAA	0	-	-	-			
	25	-	-	-			
	50	-	P	-			
	100	-	-	-	-	-	
	200	-	-	-	-	-	-

P denotes presence of *Hear*NPV detected for a given sample through PCR.

Table 3.3: Arthropods across seven orders and eighteen families were observed in contact with *Helicoverpa armigera* Nucleopolyhedrovirus-infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae through both physical and sentinel observations in the fields manually infested with infected *H. zea* larvae.

Physical Observations

Order	Family	Species	Common Name
Diptera	Milichiidae		
	Ulidiidae	*Rivellia quadrifasciata*	Soybean Nodule Flies
	Sarcophagidae		Flesh Flies
	Tachinidae		
Hemiptera	Miridae	*Lygus lineolaris*	Tarnished Plant Bugs
	Pentatomidae	*Acrosternum hilare*	Green Stink Bugs
	Reduviidae*		Assassin Bugs
	Nabidae*		Damsel Bugs
	Geocoridae*	*Geocoris* sp.	Big-eyed Bugs
	Membracidae	*Spissistilus festinus*	Three-cornered Alfalfa Hoppers
Hymenoptera	Vespidae	*Polistes* spp.	Paper wasps
	Formicidae		Ants
Coleoptera	Carabidae	*Lebia* sp.	Ground Beetles
	Coccinellidae	*Coleomegilla maculata*	Spotted Lady Beetles
	Chrysomelidae	*Diabrotica undecimpunctata*	Spotted Cucumber Beetles
Neuroptera	Chrysopidae		Green Lacewing Larvae
Lepidoptera	Noctuidae*	*Helicoverpa zea*	Corn Earworms

Sentinel Observations

Diptera	Ulidiidae	*Rivellia quadrifasciata*	Soybean Nodule Flies
	Sarcophagidae		Flesh Flies
	Tachinidae		
Hemiptera	Miridae	*Lygus lineolaris*	Tarnished Plant Bugs
Orthoptera	Tettigoniidae		Katydids

*Denotes a previously identified carrier of *Hear*NPV.

Table 3.4: All arthropods confirmed as facultative carriers of *Helicoverpa armigera* Nucleopolyhedrovirus through PCR from the first sample date (3 days after infestation), and their distribution and frequency for all sampled areas, as well as the total number of positive samples for each carrier across all fields where *Hear*NPV-infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) were manually infested.

Distances (ft)	TPB[1]	Flies[2]*	Spiders[3]	Wasps[4]**ψ	SNF[5]	TCAH[6]	BEB[7]	SCB[8]	GCW[9]	GC[10]	GSB[11]	TB[12]	LW[13]ψ
0	1	0	0	0	1	0	0	0	0	0	1	0	0
25	1	0	0	0	0	0	0	0	0	1	0	0	0
50	0	0	1	0	0	0	0	0	1	0	0	0	1
100	1	0	1	0	1	0	0	0	1	0	0	1	0
200	3	5	3	5	2	2	2	2	0	0	0	0	0
Total	6	5	5	5	4	2	2	2	2	1	1	1	1

[1]Tarnished plant bug (n=13), [2]Flies (n=18), [3]Spiders (n=19), [4]Wasps (n=3), [5]Soybean nodule fly (n=6), [6]Three-corner alfalfa hoppers (n=20), [7]Big-eyed bug (n=7), [8]Spotted cucumber beetle (n=10), [9]Green cloverworm (n=22), [10]Grape colaspis (n=19), [11]Green stink bug (n=8), [12]Tiger beetle (n=1), [13]Green lacewing larvae (n=2),.

Ψ Denotes association ($p<0.05$) with positive PCR samples by using Fisher's Exact Test, ($\alpha=0.05$).

*Flies include samples from Calliphoridae, Sarcophagidae, and Tephritidae.

**Wasps include samples from Vespidae and Sphecidae.

Figure 3.1: Soybean nodule fly (*Rivellia quadrifasciata* Macquart (Diptera: Ulidiidae)) feeding on a liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.2: Miridae (Hemiptera) approaching a liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva after being startled off.

Figure 3.3: A tarnished plant bug (*Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae)) after engorging on an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.4: A big-eyed bug (Hemiptera: Geocoridae) approaching a liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.5: A three-cornered alfalfa hopper (Hemiptera: Membracidae) close to a liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.6: Ants (Hymenoptera: Formicidae) scavenging a liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.7: Ants (Hymenoptera: Formicidae) scavenging the head of a liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.8: The remains after the ants (Hymenoptera: Formicidae) have scavenged the liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva and some of the leaf material.

Figure 3.9: A reduviid species (Hemiptera: Reduviidae) consuming the liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.10: A spotted lady beetle (*Coleomegilla maculata* (Degeer) (Coleoptera: Coccinellidae)) feeding on the remains of an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.11: A green stinkbug (*Acrosternum hilare* (Say) (Hemiptera: Pentatomidae)) feeding on the remains of an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.12: A green lacewing larva (Neuroptera: Chrysopidae) feeding on the liquefied *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.13: A species of *Lebia* (Coleoptera: Carabidae) feeding on the remains of an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.14: A healthy *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva cannibalizing the remains of an infected *H. zea* larva.

Figure 3.15: A vespid (Hymenoptera: Vespidae) feeding on the remains of an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.16: Milichiidae (Diptera) feeding on the liquid from an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.17: Two flies (Diptera) facing off over the remains of an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva.

Figure 3.18: Several Milichiidae (Diptera) feeding on an infected *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larva's remains.

CHAPTER FOUR: Determining the efficacy of *Helicoverpa armigera* Nucleopolyhedrovirus in vitro

Abstract:

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) is a naturally-occurring virus commercially produced for control of Heliothines, including *Helicoverpa zea*. Previously, the main drawback with using this virus for control has been the slower time to mortality compared to synthetic insecticides; however, a formulation that is currently available appears to be more virulent. The objective of this study was to determine the efficacy of *Hear*NPV applied at a known concentration through percent mortality and defoliation for control of each larval instar, and control of a second generation. Fourteen days after the first infestation, all plants were re-infested with a second instar larva to simulate a second generation.

*Hear*NPV was effective at controlling 1^{st}-3^{rd} instars, resulting in 99% mortality over 5 days. However, 4^{th} and 5^{th} instars only reached 35% mortality. The second generation died between 3.4 and 3.8 days, significantly faster than the first generation of 2^{nd} instars which averaged 4.9 days. An increase in mortality rate is probably due to increasing viral concentrations after replicating within the host. Final defoliation percentages were significantly smaller in the sprayed plants versus the unsprayed. Third and fourth instar larvae caused percent defoliation to exceed the threshold of 40%. *Hear*NPV in the formulation of Heligen can control 1^{st}-3^{rd} instars within 5 days, while keeping defoliation below the action threshold of 40%.

Introduction:

Helicoverpa armigera Nucleopolyhedrovirus (*Hear*NPV) is a viral biopesticide specific to Heliothines such as *Helicoverpa zea*, the most damaging pest of soybean (*Glycine max* (L.) Merrill) in the Mid-South United States (Young and McNew 1994; Musser 2015a). *Hear*NPV can be applied like a typical insecticide, and is commercially available as Heligen (Agbitech Corporation, Queensland, Australia) or Helicovex (Andermatt Biocontrol Ag, Grossdietwil, Switzerland). These formulations are relatively inexpensive and can induce epizootic events through horizontal transmission (Fuxa 1989). However, there is some concern as to how quickly mortality occurs from initial infection, as previous studies investigating a different strain sold under the tradename Elcar exhibited less than desirable time to mortality, ranging from 3 to 8 days (Stacey et al. 1977a; Luttrell et al. 1982a; Luttrell et al. 1982b; Luttrell et al. 1983). Three to 8 days from application to mortality could discourage growers from implementing *Hear*NPV into their spray regimes. As with many typical synthetic insecticides, growers expect control to occur within 3 days and suspect a failed application if live insects are observed beyond 5 days. The observed virulence of Heligen and other new formulations of *Hear*NPV suggest that they could be commercially viable, as they have been observed to prevent feeding and cause mortality much faster than Elcar.

Horizontal transmission is an important factor in the efficacy of *Hear*NPV as an insecticide, as horizontal transmission is a critical part of inducing epizootic events (Fuxa 1989). Along with increasing viral infections in a localized area, horizontal transmission of *Hear*NPV lead to viral infection of sequential generations. Although previous studies found the potential for Elcar to be transmitted from one generation to the next at around 11%, these studies have not

been performed for the possibly more virulent *Hear*NPV strain currently commercially available as Heligen (Ali et al. 1985).

The first objective of this study was to determine the ability of *Hear*NPV applied at the commercially recommended rate (1.6 oz/acre) to kill each instar of *H. zea*. The second objective of this study was to determine if differences in time to mortality or time to pupation existed when different instars of *H. zea* were introduced to soybean treated with *Hear*NPV. The third objective of this study was to determine if an application of *Hear*NPV would kill a second infestation of second instar *H. zea* larvae. The fourth objective of this study was to determine if an application of *Hear*NPV would lead to a reduction in soybean leaf defoliation by exposed larvae. It was hypothesized that later instars would exhibit decreased mortality, with earlier instars dying faster than later instars. This would result in less defoliation in the earlier instars when compared to the untreated check, but not the later instars. Also, the Heligen strain of *Hear*NPV would be capable of infecting a second generation regardless of the previous instar.

Materials and Methods:

Soybeans were planted 1 May, 13 June, and 14 July, 2017, in 4in x 4in x 3.5in pots (Greenhouse Megastore, Danville, IL) using potting soil (The Scotts Miracle-Gro Company – Landscaping, Marysville, OH). The soybean cultivar used was Pioneer 47T36 (DuPont Pioneer, Johnston, IA) with no seed treatment. Pots were watered twice daily until soybeans reached the V3 growth stage. Plants were kept in a greenhouse at the Lonoke County Research and Extension Center in Lonoke, AR. Plants randomly assigned to be untreated were caged and infested before the treated plants were sprayed. Plants randomly assigned to be treated with the virus were removed for a short period of time to be sprayed with *Hear*NPV (AgBiTech

Corporation, Queensland, Australia) at a rate of 1.6 oz/acre, and returned to the greenhouse after the spray had dried. The greenhouse was kept between 72.5° and 92.0° F. Only natural light was utilized for this experiment.

A randomized complete block design with two blocking factors was utilized to determine the ability of *Hear*NPV to kill *H. zea* larvae feeding on soybean plants. The treatment arrangement was a 5 x 2 full factorial with two factors: larval instar (1^{st}-5^{th}) and application of the virus (sprayed or unsprayed). The two blocking factors were location within the greenhouse (18) and the run number (3). Each run contained all combinations of both factors, and completed in 18 areas based on location within the greenhouse. Each area consisted of 5 cages, for a total of 90 cages per run, with 60 of these cages being sprayed and 30 unsprayed treatment combinations. There were 12 replications of the sprayed treatment combinations and 6 replications of the unsprayed treatment combinations during each run. This design was utilized because the untreated plants were to verify no viral movement and to have a baseline of percent defoliation. In the first run, *Hear*NPV spread to several unsprayed larvae; wooden barriers 3' x 5' were used to separate the unsprayed from the sprayed plants for the two remaining runs.

Infestations began at the V3 growth stage for all replications, and sprayed plants received an application of Heligen at a rate equivalent to 1.6 oz/acre immediately before infestation. *Helicoverpa zea* larvae were purchased from Benzon Research Inc. (Carlisle, PA) for this experiment. Heligen was applied with a CO_2 backpack sprayer at 10 GPA, using a ground speed of 3 miles/hr. A single larva was placed in each white insect rearing cage, 8 x 16 inches (BioQuip Products, Rancho Dominques, CA). Cages were utilized to restrict the larvae from moving to other plants where virus was not present, as well as to eliminate potential cannibalism. Mortality, percent defoliation using visual estimations, and larval molting were recorded twice

daily along with time to pupation or mortality, final larval instar, planting date, agronomic inputs, and symptomology of the infected larvae. Fourteen days after the first infestation, each original sleeve cage was re-infested with a healthy 2nd instar larva to determine the efficacy of each instar in cross-generational infection. For the purpose of this study, cross-generational infection refers to the ability of *Hear*NPV to move across time from one larval population to the next. The same monitoring protocol was used as in the first infestation.

Data concerning the final fate of each larvae, pupation or mortality, was subject to an ANOVA ($\alpha = 0.05$), and Fisher's Exact Test, as the response variable was considered categorical. Main effects consisted of larval instar, treatment, and interaction effects between larval instar and treatment. Block number and run number were analyzed as random effects. To determine differences in the time to mortality an ANOVA ($\alpha = 0.05$) which utilized a Tukey's post hoc analysis was used. Defoliation percentages were subjected to an ANOVA ($\alpha = 0.05$) which utilized Tukey's post hoc analysis. Mortality of the second generation was subject to an analysis of variance with an alpha value of 0.05 that utilized Tukey's post hoc analysis. All data was analyzed using SAS 9.4 (PROC GLIMMIX. Version 9.4, SAS Institute Inc., Cary, NC).

Results:

For the sprayed plant treatment combinations, 1st, 2nd, and 3rd instar larvae had the highest mortality (Table 1). Only one 1st instar larva survived to pupation. Time to mortality in the first infestation ranged from 4.5 to 6 days, and averaged 5 days (Table 1). There was no difference in time to mortality for 1st, 2nd, or 3rd instars. There was a significantly shorter time to mortality in the 1st instars (4.7 days) compared to 4th instars (6.2 days), and only 35 percent of the 4th instars were killed, compared to almost 100 percent of the 1st, 2nd, and 3rd instar larvae. Only one 5th

instar larva that was introduced to a sprayed plant died from the virus, and all others pupated (Table 1). Mortality from the virus was observed in 28% of the unsprayed larvae, with 2nd instar larvae having the most viral mortality, even when they were separated by barriers within the greenhouse. When unsprayed larvae died due to infection by the virus, mortality took anywhere from 7.5 to 11.3 days, implying that they were infected from the liquification of the sprayed larvae (Table 1).

When added to previously sprayed plants, the second infestation resulted in 100% mortality, and averaged 3.5 days until mortality occurred. The time to mortality for the sprayed plants had no significant differences between instars (Table 1). The unsprayed plants also had mortality in the second infestation due to the movement of the virus across the cages. The unsprayed plants had a shorter time to death when added to plants that previously contained a 1st instar than when added to plants that previously contained a 5th instar larva, ranging from 3.9 to 6.5 days to mortality respectively (Table 1).

Unsprayed plants averaged a final defoliation percentage of 40% across all instars, which is below the current defoliation threshold for soybean in Arkansas (Figure 1), while sprayed plants ranged from 3% to 39%, with an average of 19.6%. Plants sprayed with *Hear*NPV had significantly less ($p < 0.001$) defoliation than unsprayed plants (Figure 1). In both sprayed and unsprayed treatments, 4th instar larvae caused significantly more defoliation than all other instars except 3rd instar larvae (Table 1). First, 2nd, and 5th instar larvae on unsprayed plants exhibited defoliation percentages of 26%, 15%, and 23% respectively, with all but 1st instar being significantly lower than 3rd instar larvae at 51%. For the sprayed plant treatment combinations, the 3rd and 4th instar larvae had significantly more defoliation than all other larval instars except 5th instar larvae, but exhibited significantly less defoliation than was seen in larvae on unsprayed

plants. First and 2nd instars exhibited significantly less defoliation than all other instars, but did not differ between themselves. In the unsprayed treatment, 3rd and 4th instar larvae exhibited defoliation above the action threshold, while only 4th instar larvae on sprayed plants exhibited defoliation above the threshold (Table 1).

Discussion:

The *Hear*NPV application caused mortality to occur within 4.5 and 5.5 days for 1st–3rd instars, showing control for the target population would take approximately 5 days. This is within the range of time to mortality reported by Luttrell et al. (1982a), where control of *H. zea* larvae with *Hear*NPV took from 3 to 8 days, suggesting no increase in virulence in the current formulation of *Hear*NPV. In the second generation the average time to mortality was 3.5 days across the sprayed plots regardless of previous larval instar. These findings are corroborated by Ali et al. (1985), where a population of infected larvae were found to infect the sequential population. If compared to the time to mortality for the first generation of 2nd instar larvae, there is a significant decrease in how long the virus takes to kill. Larvae in the second infestation took less time to die than in the first infestation, indicating a potential increase in virulence between generations. An increase in virulence between generations is most likely due to the highly concentrated release of virus occlusion bodies in the rupturing of the infected larva's cuticle, previously explored by Ali et al. (1987).

An application of *Hear*NPV reduced defoliation caused by all instars. This is supported by Alam et al. (1987), where 1st, 2nd, and 3rd instars fed less when infected with *Hear*NPV. This is likely due to *Hear*NPV killing larvae before they are able to finish their larval life, rather than appetite suppression. Across treatments there was a trend for the later instars to cause increased

defoliation, which supports work by McWilliams (1983) and Flusche et al. (1986). However, 5th instar larvae were observed to feed significantly less than the 4th instars. It is possible that 5th instar larvae consumed the majority of their needed diet for pupation prior to the initiation of the study, which is supported by the rapid onset of pupation in 5th instar larvae. When comparing defoliation percentages by instar across treatments, there is a significant reduction in defoliation for all instars except the 5th instar when an application of *Hear*NPV was made, which correlates with the work done by Alam et al. (1987). For plants that were not sprayed, all introduced were expected to cause defoliation above the action threshold of 40%. However, due to contamination of *Hear*NPV on the unsprayed plants, a large amount of 1st and 2nd instar larvae exhibited mortality from *Hear*NPV. Third and 4th instar larvae exhibited little mortality and caused over 50% defoliation, well above the Arkansas defoliation threshold for soybean of 40%. When contrasted to the sprayed treatment, only 4th instar larvae were close to the action threshold at 39% defoliation. These results confirm that the target population for control in a soybean field should be mainly 1st-3rd instar larvae, as 4th instar larvae could still cause significant defoliation before death. Alam et al. (1987) and Luttrell et al. (1982a) also found that 1st-3rd instar larvae should be targeted by *Hear*NPV.

An application of *Hear*NPV provided adequate control for 1st, 2nd, and 3rd instar larvae, resulting in their death approximately 5 days after the application. This application is also capable of keeping the defoliation percentage below the economic threshold. However, *Hear*NPV in the form of Heligen does not provide adequate control of 4th or 5th instars before significant defoliation occurs. *Hear*NPV can provide control of sequential generations when adequate amounts of virus remain; however, it is likely that many environmental factors leading to degradation of *Hear*NPV were not captured by this study. It was also found that the second

generation died faster than the first, probably due to viral concentrations and low UV degradation. Future studies should explore cross-generational interactions and look to include UV light as a controllable factor.

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Table 4.1: Determining the efficacy of *Helicoverpa armigera* Nucleopolyhedrovirus by investigating mortality, pupation, time to mortality/pupation, and percent defoliation across *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larval instars when applied to V3 soybeans, and the time to mortality of a simulated second generation infested 14 days after the application.

Treatment	Instar	% Mortality*	% Pupated*	Days to Mortality**	Days to Pupation**	2nd Infestation Days to Mortality**	% Final Defoliation**
Unsprayed	1	46 b	54 b	11.3 a, ψ	12.7 a, ψ	3.9 b, ψ	26 bc, ψ
	2	92 a	8 c	7.4 a, ψ	12.5 a	4.7 ab, ψ	15 c
	3	38 b	62 b	7.5 ab	11.1 a	4.9 ab	51 ab, ψ
	4	12 bc	88 ab	9.5 ab	6.1 ab	5.9 ab	54 a, ψ
	5	0 c	100 a	-	4.4 b	6.5 a	23 c
Sprayed	1	97 a	3 c	4.7 b, ψ	14 b, ψ	3.7 a, ψ	3 c, ψ
	2	100 a	0 c	4.9 ab, ψ	-	3.8 a, ψ	8 c
	3	100 a	0 c	5.5 ab	-	3.5 a	25 ab, ψ
	4	35 b	65 b	6.2 a	5.3 a	3.4 a	39 a, ψ
	5	3 c	97 a	4.5 c	4.5 a	3.5 a	23 b

Lowercased letters denote a significantly different value within the sprayed or unsprayed treatments.
* Denotes statistical analysis according to a Fisher's Exact Test (α=0.05).
** Denotes statistical analysis according to a Tukey's LSD (α=0.05).
ψ Denotes a value that is significantly different within an instar between treatments.

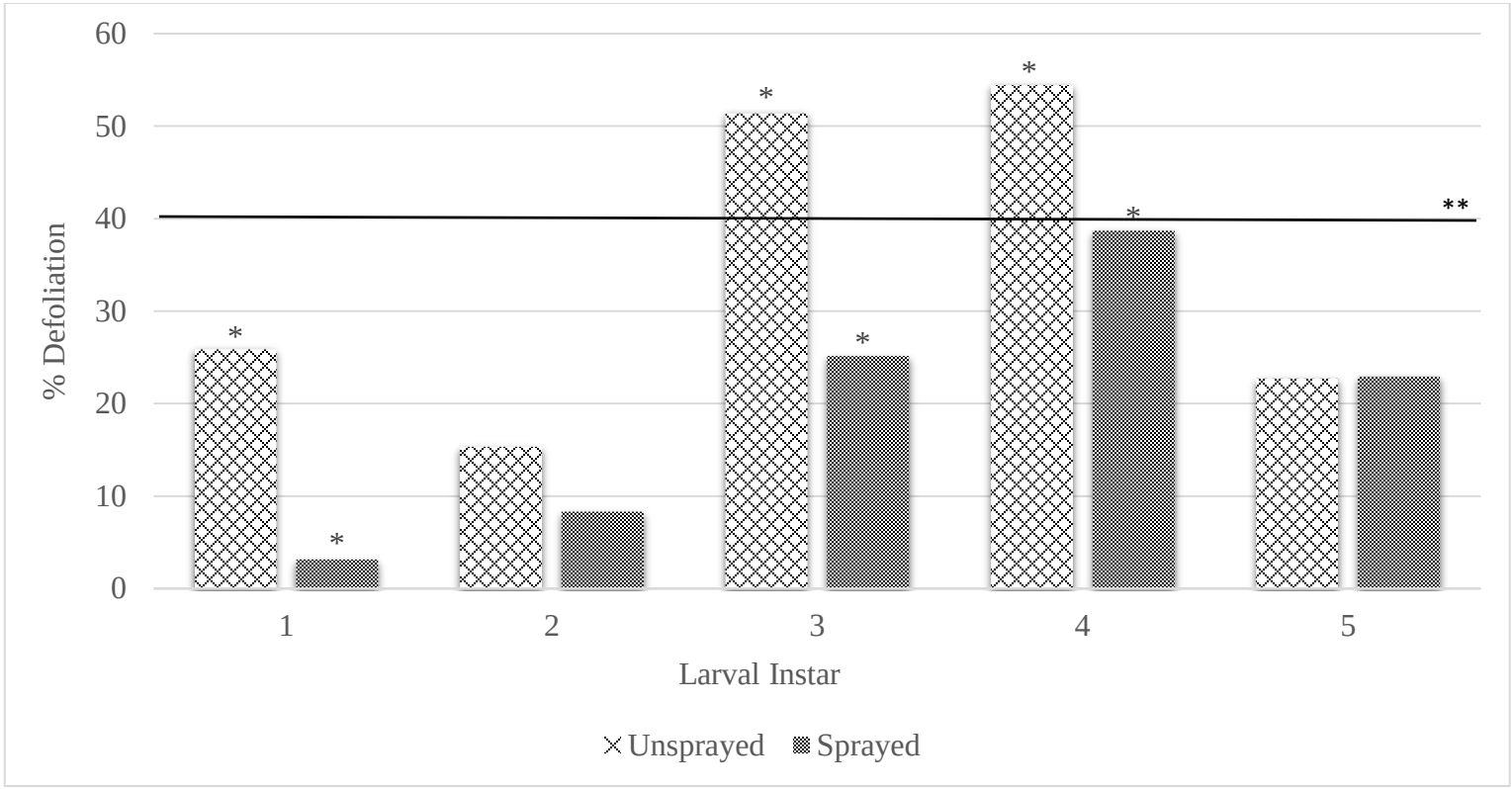

Figure 4.1: Determining the efficacy of *Helicoverpa armigera* Nucleopolyhedrovirus versus the untreated through percent defoliation across *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larval instar when applied to V3 soybeans compared to the economic threshold of 40%.
* Denotes significant differences for that instar across treatments using ANOVA (α=0.05) and a Tukey's post hoc analysis (p=0.05).
** Denotes the action threshold of 40% defoliation.

Conclusion:

This study identified several arthropods as novel carriers of *Hear*NPV. They were either obligatory or facultative carriers. Obligatory carriers were those that were coated in *Hear*NPV through an application, while facultative carriers were those observed contacting infected larvae. Novel obligatory *Hear*NPV carriers identified were: *H. zea* larvae (Noctuidae), three-cornered alfalfa hoppers (Membracidae), yellow-striped armyworms (Noctuidae), spiders (Araneae), big-eyed bugs (Geocoridae), nabids, green cloverworms (Erebidae), grape colaspis (Chrysomelidae), bean leaf beetles (Chrysomelidae), spotted cucumber beetles (Chrysomelidae), and short-horned grasshoppers (Acrididae). These arthropods were carriers of *Hear*NPV 2 days after the application, and some were capable of disseminating *Hear*NPV 200 feet from the application area. Novel facultative *Hear*NPV carriers identified were: milichiid flies, sarcophagid flies, tachinid flies, soybean nodule flies (Ulidiidae), tarnished plant bugs (Miridae), green stink bugs (Pentatomidae), three-cornered alfalfa hoppers (Membracidae), paper wasps (Vespidae), ants (Formicidae), *Lebia* sp. (Carabidae), spotted lady beetle adults (Coccinellidae), spotted cucumber beetles (Chrysomelidae), green lacewing larvae (Chrysopidae), and katydids (Tettigoniidae). Previously identified carriers were also observed including *H. zea* larvae, reduviids, nabids, and big-eyed bugs (Geocoridae). These facultative carriers were carriers of *Hear*NPV 3 days after the field infestation, with some capable of disseminating *Hear*NPV 200 feet from the infestation area. These novel arthropod carriers could be important in the induction of an epizootic event.

*Hear*NPV was present in the crop canopy between 13 and 21 days after an application was made, revealing a residual time of around 13 days. When rolling populations of *H. zea* were observed, *Hear*NPV was able to disseminate and remain active longer than in fields with declining *H. zea* populations. Therefore, this bio-insecticide could be more effective against

rolling populations rather than a single generation. *Hear*NPV does seem to be a viable bio-insecticide for fields infested with 1st through 3rd instar larvae, but not for fields infested with later-stage larvae. Also, growers should understand that mortality will take between 3 to 8 days with this product; therefore, an application should be made before the action threshold is reached.