Journal of Economic Entomology, XX(XX), 2019, 1–7
doi: 10.1093/jee/toz012
Research

Field Studies on the Horizontal Transmission Potential by Voluntary and Involuntary Carriers of *Helicoverpa armigera nucleopolyhedrovirus (Baculoviridae)*

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Subject Editor: Denny Bruck

Received 26 September 2018; Editorial decision 4 January 2019

Abstract

Horizontal transmission of *Helicoverpa armigera nucleopolyhedrovirus* (HearNPV) has been found to occur through several pathways involving abiotic factors such as soil, wind, and rain, and biotic factors such as predators, parasitoids, and infected hosts. Previous studies examining horizontal transmission through certain biological carriers speculated they were likely not significant in increasing infection rates, however; these studies only focused on a relatively small number of arthropods present within a field setting. This study was conducted to evaluate the horizontal transmission potential of HearNPV by all potential biological carriers when applied as a foliar bioinsecticide or as virus-infected, nonmotile *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) larvae in a soybean field. Soybean plots were either sprayed with HearNPV or infested with late-stage HearNPV-infected larvae, and sample zones were sampled 3, 7, 10, 14, 17, and 21 days after the infestation, and analyzed for viral presence using PCR. We then identified HearNPV carriers through contamination from the application (involuntary) or through contact with a HearNPV-infected larva (voluntary). Both were confirmed through PCR analysis. Regardless of application technique, on average, HearNPV was capable of disseminating up to 61.0 m in 3 d after inoculation and was found within the sampled canopy 13–21 d after inoculation. Several arthropods were identified as novel carriers of HearNPV. Results from this study indicate that many novel HearNPV carriers are likely important in disseminating HearNPV.

Key words: *Helicoverpa armigera nucleopolyhedrovirus*, horizontal transmission, *Helicoverpa zea*, entomopathogen

Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) is a major pest of several crops, and is the most damaging pest of soybean (*Glycine max* (L.) Merrill) in the Mid-South United States (Musser et al. 2017). Once *H. zea* populations within a field reach economic threshold, an insecticidal application is warranted, with an average of 1.09 applications per acre occurring across seven southern states in 2016 (Musser et al. 2017). However, *H. zea* has developed resistance to many insecticide classes once commonly used for control, including: organochlorides, organophosphates, and pyrethroids (Brazzel 1963, Graves et al. 1963, 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, Musser et al. 2015). Considering that *H. zea* has an affinity for developing resistance, there is a need for new control tactics, especially those that are effective, economical, and offer low health risks to the applicator.

Helicoverpa armigera nucleopolyhedrovirus (HearNPV) is a virus in the family *Baculoviridae* that is specific to Heliothines. HearNPV 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 can occur through several routes. Abiotic conditions such as rainfall and wind can transport occlusion bodies from the soil to the immediate crop canopy where infection can

occur, however; dissemination in this method seldom yields farther up the plants than the understory of the crop canopy (Young 1990; Fuxa and Richter 2001, 2006).

Several biotic factors have been studied to evaluate the potential impact on horizontal transmission; these factors include cannibalism, surface contamination by infected larva, parasitism, and predation. Infected larvae are capable of transmitting HearNPV through cannibalism by a healthy larva (Vasconcelos 1996). Infected larvae can defecate viral particles in adequate concentrations to initiate infection when consumed, or they can spread HearNPV through surface contamination by vomiting, liquefaction, or movement (Ali et al. 1987a,b; Vasconcelos 1996). Parasitoids, such as *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), transmit HearNPV 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, 1990a). Predators including *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 HearNPV (Young and Yearian 1990b). *Nabis roseipennis* Reuter can feed on an infected larva and, 10 d after feeding, defecate frass that contains a viral concentration high enough to induce infection when ingested (Young and Yearian 1987). However, most studies found these biotic factors to have a minimal role in the development of a HearNPV epizootic event (Ali et al. 1985; Young and Yearian 1989, 1990a,b; Vasconcelos 1996; Fuxa and Richter 2006).

Several studies have revealed the potential for certain arthropods to transmit HearNPV; however, no studies have explored the potential of dissemination by nonparasitic or nonpredatory arthropods. A more effective application of HearNPV could 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. The study evaluated the horizontal transmission potential of HearNPV by biotic carriers in soybean fields by identifying the dissemination potential when HearNPV was applied as a bioinsecticide, or was applied as infected, nonmotile *H. zea* larvae. Horizontal transmission potential was also evaluated by identifying carriers of HearNPV that were either sprayed during the application (involuntary carriers) or observed in contact with infected larvae (voluntary carriers). The arthropods that were sprayed during the application were termed 'involuntary carriers' because they were anthropogenically forced to carry HearNPV, while those that were observed in contact with an infected larva were termed 'voluntary carriers' since no anthropogenic force caused their contamination. Two application methods were utilized in an attempt to distinguish the dissemination potential of each class of carriers, involuntary and voluntary. The spray application initially resulted in only involuntary carriers, because the only source of HearNPV was from the application, and no larvae infected from the spray should have died by the first sample date. However, soon after the first sample date, both voluntary and involuntary carriers would be present as host larvae died. In the fields infested with moribund larvae, involuntary carriers were excluded, since no arthropods were forced to carry HearNPV, and only voluntary carriers were present. We hypothesized that the potential for horizontal transmission would be high, due to the lack of confirmed arthropods as carriers.

Materials and Methods

Virus and Insects

The HearNPV used in all experiments was a commercial formulation with a concentration of 7.5×10^9 occlusion bodies (OBs)/ml in a liquid suspension (Heligen, AgBiTech LLC, Fort Worth, TX). The HearNPV was stored at 4°C until used. *Helicoverpa zea* larvae used to inoculate fields were acquired from Benzon Research (Carlisle, PA) as second instars.

Field Setup

Each field study used a similar plot setup. An area of 15.2 m by 15.2 m located on the edge of a soybean field served as the HearNPV-inoculation area. The remaining portion of each field was left uninoculated, and sample distances of 0–7.6, 7.6–15.2, 15.2–30.5, and 30.5–61.0 m from the inoculation area were established and flagged with 91.4 cm tall wire flags (Gempler's, Janesville, WI) prior to the release of HearNPV in the inoculation area. All samples were collected with a standard 38.1 cm mesh sweep net (BioQuip Products, Rancho Dominques, CA) swung through the canopy perpendicular to the row. During sampling, at least one step was taken between each sweep to ensure 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 partially sampled. During each sweep, the top of the sweep net contacted the crop under the canopy approximately midway up the stem to ensure arthropods present would fall into the net. Although this method of collecting could cause some cross-contamination within the subsamples, there was no better sampling procedure available. Additionally, the PCR primers were capable of detecting occlusion body concentrations as low as 750 OBs/ml. This amount of cross-contamination was not probable and was further reduced by quickly placing the samples in a –20°C freezer to reduce arthropod movement. Samples were only handled when analysis began. Once a field was selected, three samples consisting of 10 sweeps each were taken prior to the inoculation of HearNPV to verify that no natural HearNPV was present in each field. Also, three soil samples were taken for each field, and analyzed using the published protocol described by Evans et al. (1980). The only modification was instead of examining the soil samples under oil immersion, we conducted DNA extraction and PCR analysis as described for all other samples in both experiments. Four fields were utilized for experiment 1, and three fields were utilized for experiment 2.

Experiment 1: HearNPV Applied as a Bioinsecticide

HearNPV was applied in four soybean fields near Lonoke, AR (field 1–4). The virus was applied at a rate of 8.76×10^{11} OBs/ha (116.8 ml Heligen/ha) using a CO_2 backpack sprayer applying a spray volume of 93.6 liter/ha using a ground speed of 4.8 km/h. Field 1 was treated on 5 August 2016, field 2 and field 3 were treated on 7 August 2017, and field 4 was treated on 21 July 2017. Throughout the experiment, precautions were taken to minimize anthropogenic movement. During the application, the applicator did not leave the inoculation 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 toward the inoculation area. All samples for one distance were taken sequentially, and then the sampler acquired a non-exposed sweep net and moved to the adjacent sampling area.

In 2016, field 1 was sampled 3, 7, 14, and 21 days after the application (DAA). Three samples, each consisting of 10 sweeps, were taken for each distance, including the application area, for each sample date. In 2017, field 2 and field 3 were sampled 2, 6, 9, 13, and 20 DAA, and field 4 was sampled 3, 7, 10, 14, and 21 DAA. Each sample date consisted of three samples from the application area and three from the 0–7.6 m area at 10 sweeps, three samples from the 7.6–15.2 m area at 12 sweeps, five samples from the 15.2–30.5

m area at 21 sweeps, and six samples from the 30.5–61.0 m area at 58 sweeps, for a total of 20 samples per sample date. Because the further sample zones were larger in area, this increase in sample size allowed for equivalent proportions of each sample zone to be sampled. All samples were transferred to a −20°C freezer as quickly as possible to reduce within-sample contamination, and frozen for a minimum of 48 h to ensure arthropod mortality.

Experiment 2: Horizontal Transmission Potential From HearNPV-Infected Larvae

Helicoverpa zea larvae were allowed to feed on diet contaminated with 100 µl of a solution containing HearNPV at a concentration of 7.8×10^4 OBs/ml for 48 h once they reached the third instar, and then were observed for symptoms of infection prior to use. Five to eight days after inoculation, *H. zea* larvae that were moribund and nonmotile were manually placed into the HearNPV-inoculation areas (field 5–7). In 2016, field 5 was infested on 4 August with 90 infected *H. zea* larvae, which correlated to 1 larva per 1.2 row-meter. Samples were taken 3, 7, 14, and 21 days after infestation (DAI). Three samples, each consisting of 10 sweeps, were taken for each distance, including the application area, for each sample date. In 2017, two fields were infested, field 6 on 19 June and field 7 on 19 July, at a rate of 1 larva per 0.61 row-meter. Field 6 was infested with 283 larvae, and sampled 3, 7, 14, 17, and 21 DAI. Field 7 was infested with 410 larvae, and sampled 3, 7, 10, 14, and 22 DAI. This variation in infestation numbers was due to a shorter row spacing in field 7, resulting in more larvae in a given area. Each sample date for the 2017 field season consisted of three samples from the inoculation area and three from the 0–7.6 m area at 10 sweeps each, three samples from the 7.6–15.2 m area at 12 sweeps each, five samples from the 15.2–30.5 m area at 21 sweeps each, and six samples from the 30.5–61.0 m 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 were transferred to a −20°C freezer as quickly as possible to reduce within-sample contamination, and frozen for a minimum of 48 h to ensure arthropod mortality. Infected larvae within the inoculation area were monitored using Bushnell NatureView trail cameras (Bushnell Corporation, Overland Park, KS) and manual observations in order to observe arthropod species that were possible carriers of HearNPV.

Sample Analysis

Arthropods present in each individual sample were identified and quantified. Then, all pooled arthropods for a single sample were placed into a 15 ml test tube and homogenized. In 2017, each of the samples taken during the first sample date was further divided into five subsamples and homogenized separately. In experiment 1, these subsamples consisted of the four most abundant arthropods for a given sample, and all remaining arthropods in a single pooled sample. In experiment 2, subsamples consisted of the four arthropod species present that were most commonly observed as potential carriers within a given sample, and all remaining arthropods in a single pooled sample. OBs were extracted from the homogenized sample using a published extraction technique (O'Reilly et al. 1992) which was modified such that extraction did not continue with a sucrose gradient, and homogenization did not occur with a conventional homogenizer, but rather a sterilized glass rod. The homogenized suspension was washed with 1× phosphate-buffered saline (PBS) (10 mM phosphate, pH 7) and centrifuged at 14,000 × *g* for 20 min. The pellet was resuspended in 0.5% sodium dodecyl sulfate (SDS), centrifuged and resuspended in 0.5 M NaCl. After a final spin, the OB pellet was resuspended in 1× PBS and stored at −20°C. Any viral DNA present was extracted from the OBs with a DNA extraction kit, DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), and stored at −20°C. Extracted viral DNA was amplified with HearNPV polyhedrin-specific primers HzSpolh-2F (5′-CCCTACTTTGGGCAAAACC-3′) and HzSpolh-2R (5′-TCGGTTTGGTTGGTCGCATA-3′) (IDT, Coralville, IA) using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). Thermocycler conditions used were 5 min of initial denaturation at 95°C; 30 cycles of amplification (30 s at 95°C, 30 s at 57°C, and 60 s at 72°C); a 3 min final extension at 72°C; and a hold at 4°C. All PCR mixtures (50 µl) contained 1 µl extracted DNA sample, 0.2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer, 1× GoTaq Flexi Buffer, and 1.25 U of GoTaq Hot Start DNA polymerase (Promega, Madison, WI). A positive control of Heligen and a negative control of deionized water were included in each thermocycler run to confirm successful amplification of the target gene. After the amplification of DNA by PCR, samples were subject to gel electrophoresis, where a 20 µl aliquot of each sample was loaded into individual wells. The PCR product was separated on a 2% agarose gel subjected to electrophoresis for 1 h at 90 V and visualized with Sybr Safe DNA gel stain (Life Technologies Corporation, Carlsbad, CA) which caused the product to fluoresce in the presence of an ultraviolet light (UPV LLC, Upland, CA). If a band was present at 450 bp, HearNPV was considered positive for that corresponding sample. In 2016, PCR products were sequenced (Eurofins, Louisville, KY), and HearNPV polyhedron sequence was confirmed, however; in 2017 the high volume of samples did not allow for this verification process.

Prior to the initiation of these experiments, arthropods were collected from uninoculated soybean fields. These arthropods were separated into the same groups used for later samples and analyzed using the same process. Each group was emulsified individually and divided in half. One half was analyzed with deionized water, and the other was analyzed with HearNPV added at a concentration of 7.8×10^6 OBs/ml. This was to determine if any interference would occur between the primers and arthropods analyzed, resulting in false negatives or false positives. All groupings were negative for HearNPV when deionized water was added, and positive for HearNPV when undiluted HearNPV was added, demonstrating the lack of interference with the PCR reaction.

All data were analyzed with an ANOVA ($\alpha = 0.05$) in SAS 9.4 (PROC GLIMMIX. Version 9.4, SAS Institute Inc., Cary, NC). Fixed effects consisted of field, DAA/DAI, and distance. Year was considered a random effect. Fixed effects for Fisher's exact test to determine associations were interaction effects between presence of each arthropod and presence of HearNPV, and number of each arthropod and presence of HearNPV.

Results and Discussion

Experiment 1: HearNPV Applied as a Bioinsecticide

Involuntary carriers and host populations

Fields utilized in these experiments were sprayed with a known concentration of HearNPV, which was determined to be the only source of HearNPV within the field by taking soil samples and sweep samples before the application of HearNPV was made. Therefore, any arthropods determined positive for HearNPV were assumed to be either involuntary or voluntary carriers. Previous studies have shown HearNPV takes between 3 and 8 d from infection to cause mortality (Luttrell et al. 1982); therefore, if carriers were identified at the 3 DAA sample date, those could be assumed to have contacted the

virus during the application, rather than contacted the virus voluntarily. After the 3 DAA sample date, positive samples could have been comprised of involuntary or voluntary carriers.

Where HearNPV was applied as a bioinsecticide, *H. zea* larvae were the most commonly identified arthropod across all fields. *Ceresa festina* (Say) (Hemiptera: Membracidea) was the second, and *Colaspis brunnea* (Fabricius) (Coleoptera: Chrysomelidae) adults were the third most commonly identified arthropod. *Hypena scabra* (Fabricius) (Lepidoptera: Erebidae) were the fourth most commonly identified arthropods. Other groups included Diptera, which comprised flies from Sarcophagidae, Calliphoridae, Tachinidae, Muscidae, and Tephritidae, spiders (Araneae), *Geocoris* spp., and *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae). It is important to note, we grouped certain arthropods into families rather than genera or species mainly because of low numbers within the samples, but in some cases because identification to species was unnecessary and complex.

When the subsamples from the first sample date of each field inoculated in 2017 were analyzed, *C. festina* was positive for HearNPV more often than any other arthropod present, with 21 ($n = 44$) subsamples returning positive (Table 1). The positive subsamples were from all distances sampled, implying that *C. festina* was acting as an involuntary carrier (due to timing from application), and capable of disseminating HearNPV at least 61.0 m. *Helicoverpa zea* and *Geocoris* spp. were positive in 13 ($n = 58$), and 12 ($n = 26$) subsamples, respectively, with infected *H. zea* larvae present in all distances while *Geocoris* spp. were present in all but the inoculation area. Spiders were positive for HearNPV in nine ($n = 26$) subsamples across three distances: 7.6, 30.5, and 61.0 m. These four arthropods accounted for 74% of the positive subsamples. The other 26% of the positive subsamples were comprised of *Diabrotica undecimpunctata* (Linnaeus) (Coleoptera: Chrysomelidae) in four ($n = 13$) subsamples, *Spodoptera ornithogalli* (Guenee) (Lepidoptera: Noctuidae) in four ($n = 10$) subsamples, *H. scabra* in five ($n = 23$) subsamples, *C. brunnea* in two ($n = 2$) subsamples, *Cerotoma trifurcate* (Forster) (Coleoptera: Chrysomelidae) in two ($n = 6$) subsamples, and grasshoppers within Acrididae and Tettigoniidae were positive for HearNPV in two ($n = 10$) subsamples (Table 1). Only subsamples comprised of individual species are represented here, pooled subsamples, and samples taken after the first sample date were not used to determine involuntary carriers.

Dissemination and Residual Presence of HearNPV

HearNPV dissemination occurred extensively when applied as a bioinsecticide. In field 1, HearNPV was observed 30.5 m from the inoculation area 7 DAA and remained in the sampled area for 14 d. All remaining fields had HearNPV-positive samples 61.0 m from the inoculation area 2–3 DAA. The time HearNPV remained in the sampled area was variable by field but ranged from 13 to more than 21 DAA. This variability in residual presence could be due to *H. zea* population variation between fields (Tables 2–4). *Helicoverpa zea* populations in field 1 had a significant two-way interaction between sample dates and sample distances, with significantly larger populations in the application area and the 7.6 m sample area 3 DAA than any other distance on any other date (Table 2). There was also an overall trend for populations to decrease until 14 DAA, at which point, they were no longer significantly different from the 21 DAA sample date. Field 2, field 3, and field 4 *H. zea* populations had a one-way interaction with DAA and a one-way interaction with distance, but no two-way interaction was determined (Tables 3 and 4). Field 2 and field 3 had a decrease in *H. zea* populations as DAA increased (Table 3), and field 2 and field 3 had significantly lower populations outside the application area (Table 4). Only field 4 had a *H. zea* population above the economic threshold for the entire study, and it was the only field where an epizootic was observed by the sampler (Tables 3 and 4). All other fields had larval populations close to the economic threshold when the study was initiated, but those populations were reduced to much lower levels within 14 DAA (Tables 2–4). The population reduction could have been associated with presence of HearNPV and other natural enemies, or simply the maturation of that generation, with no sequential infestation. When presence of *H. zea* larvae was analyzed with HearNPV presence using Fisher's exact test, presence of HearNPV was found to be nonindependent of *H. zea* presence for all fields. Therefore, HearNPV residual presence within a soybean field does appear to be influenced by presence or absence of host populations. This implies an application of HearNPV would not be as beneficial if applied as a preventative measure but would maximize horizontal transmission potential by being applied when the host was present, and continual generations of the host infested the area.

Experiment 2: Horizontal Transmission Potential From HearNPV-Infected Larvae

Voluntary carriers

The fields utilized in this study were infested with a number of HearNPV-infected, nonmotile *H. zea* larvae. These larvae were the only source of HearNPV within the field; therefore, any arthropod found positive for HearNPV after PCR analysis is assumed to have contacted

Table 1. All arthropods confirmed with PCR as involuntary carriers of HearNPV 3 DAA

		No. positive for HearNPV						
		Distance (m)						
Arthropods	Sample size (n)	0	7.6	15.2	30.5	61.0	Total	% Carriers
Ceresa festina	44	4	3	2	5	7	21	47.7
Helicoverpa zea	58	5	1	2	1	4	13	22.4
Geocoris spp.	26	0	1	2	4	5	12	46.2
Araneae	26	0	2	0	3	4	9	34.6
Diabrotica undecimpunctata	13	1	0	0	0	3	4	30.8
Spodoptera ornithogalli	10	2	0	0	1	1	4	40.0
Hypena scabra	23	4	0	0	1	0	5	21.7
Colaspis brunnea	2	0	0	0	0	2	2	100
Cerotoma trifurcate	6	0	1	0	0	1	2	33.3
Grasshoppers (Acrididae and Tettigoniidae)	10	0	0	0	1	1	2	20.0
Total	218	16	8	6	16	28	74	33.9

Table 2. Number of *Helicoverpa zea* larvae in 25 sweeps for field 1 across sample dates and distances (α = 0.05; df = 12,59; *F*-value = 2.72; *P* = 0.0087)

Field 1			
Date	DAA	Distance (m)	Mean *H. zea* (±SEM[a])/25 sweeps
8 Aug. 2016	3	0	55.0 (9.0)a
8 Aug. 2016	3	7.6	48.3 (3.6)ab
8 Aug. 2016	3	15.2	23.3 (1.7)d
8 Aug. 2016	3	30.5	20.0 (7.6)d
8 Aug. 2016	3	61.0	36.7 (7.1)bc
12 Aug. 2016	7	0	26.7 (1.7)cd
12 Aug. 2016	7	7.6	26.7 (11.6)cd
12 Aug. 2016	7	15.2	23.3 (3.0)d
12 Aug. 2016	7	30.5	19.2 (4.6)d
12 Aug. 2016	7	61.0	25.8 (4.2)cd
19 Aug. 2016	14	0	0.8 (0.8)e
19 Aug. 2016	14	7.6	1.7 (0.8)e
19 Aug. 2016	14	15.2	0.0 (0.0)e
19 Aug. 2016	14	30.5	3.3 (0.8)e
19 Aug. 2016	14	61.0	5.8 (1.7)e
26 Aug. 2016	21	0	2.5 (1.4)e
26 Aug. 2016	21	7.6	1.7 (0.8)e
26 Aug. 2016	21	15.2	2.5 (1.4)e
26 Aug. 2016	21	30.5	2.5 (2.5)e
26 Aug. 2016	21	61.0	2.5 (2.5)e

Letters denote a significantly different value using an ANOVA (α = 0.05), and a Tukey's post hoc analysis (*P* < 0.05).

[a]Standard error of the mean.

Table 3. Sample date evaluations of *Helicoverpa zea* larval populations evaluated at 25 sweeps for field 2, field 3, and field 4

Date	DAA	Mean *H. zea* (±SEM[a])/25 sweeps
Field 2 (*F* = 20.4, df = 4, 99; *P* < 0.01)		
9 June 2017	2	5.6 (0.7)a
13 June 2017	6	2.0 (0.4)b
16 June 2017	9	1.7 (0.7)b
20 June 2017	13	1.1 (0.3)bc
27 June 2017	20	0.4 (0.2)c
Field 3 (*F* = 33.2, df = 4, 99; *P* < 0.01)		
9 June 2017	2	6.9 (1.0)a
13 June 2017	6	1.8 (0.4)b
16 June 2017	9	0.5 (0.3)bc
20 June 2017	13	0.3 (0.2)c
27 June 2017	20	0.6 (0.4)bc
Field 4 (*F* = 5.0, df = 4, 99; *P* < 0.01)		
24 July 2017	3	9.8 (1.3)b
28 July 2017	7	13.9 (1.6)a
31 July 2017	10	12.8 (1.4)a
4 Aug. 2017	14	9.0 (1.0)b
11 Aug. 2017	21	9.4 (0.8)b

Letters denote a significantly different value using an ANOVA (α = 0.05), and a Tukey's post hoc analysis (*P* < 0.05).

[a]Standard error of the mean.

an infected *H. zea* larva, becoming a voluntary carrier. Analysis of the subsamples from the first sample date of field 6 and 7 revealed several voluntary carriers: *L. lineolaris* (6 positive out of 13 subsamples); Diptera in Calliphoridae, Sarcophagidae, and Tephritidae (5 out of 18); Hymenoptera in Vespidae and Sphecidae (3 out of 3); spiders (5 out of 19); *Rivellia quadrifasciata* (Macquart) (Diptera: Ulidiidae) (4 out of 6); *Geocoris* spp. (2 out of 7); *D. undecimpunctata* (2 out of 10); *Chinavia hilare* (Say) (Hemiptera: Pentatomidae) (1 out of 8); Cicindellinae (Coleoptera: Carabidae) (1 out of 1); chrysopid larvae (1 out of 2); *C. festina* (2 out of 20); *H. scabra* (2 out of 22); and *C. brunnea* (1 out of 19) (Table 5). Only subsamples comprised of individual species are represented here, pooled subsamples, and samples taken after the first sample date were not used to determine voluntary carriers.

Table 4. Sample distance evaluations of *Helicoverpa zea* larval populations evaluated at 25 sweeps for field 2, field 3, and field 4

Distance (m)	Mean *H. zea* (±SEM[a])/25 sweeps
Field 2 (*F* = 6.3, df = 4, 99; *P* < 0.01)	
0	0.8 (0.5)a
7.6	2.7 (0.9)b
15.2	4.2 (1.1)b
30.5	1.8 (0.4)bc
61.0	1.9 (0.4)c
Field 3 (*F* = 3.1, df = 4, 99; *P* = 0.02)	
0	3.0 (1.0)a
7.6	3.3 (1.5)b
15.2	1.4 (0.6)bc
30.5	1.7 (0.5)c
61.0	1.4 (0.5)bc
Field 4 (*F* = 5.6, df = 4, 99; *P* < 0.01)	
0	9.8 (1.3)b
7.6	15.2 (1.6)a
15.2	11.9 (1.8)a
30.5	12.1 (1.0)b
61.0	8.0 (0.9)b

Letters denote a significantly different value using an ANOVA (α = 0.05), and a Tukey's post hoc analysis (*P* < 0.05).

[a]Standard error of the mean.

Several arthropods were observed in contact with HearNPV-infected larvae and are reported here as voluntary carriers (Table 6). These voluntary carriers include species within four families of Diptera, six families of Hemiptera, two families of Hymenoptera, three families of Coleoptera, one family of Neuroptera, and one family of Orthoptera. Of these, only five families were not also identified as voluntary carriers through PCR: Milichiidae, Tachinidae, Coccinellidae, Tettigoniidae, and Formicidae. This study identified species in 21 insect families and spiders as voluntary HearNPV carriers. Five of these carriers have been previously documented (Ali et al. 1987a; Young and Yearian 1990b), but the remaining 17 families are reported here as voluntary carriers for the first time, and appear to be important in the dissemination of HearNPV (Table 6).

Dissemination and Residual Presence of HearNPV

Field 5, infested with 96 infected larvae which correlate to 1 larva per 1.2 row-meter, did not have a single HearNPV-positive sample for any sample date. This could be due to the low number of infected larvae used to inoculate the inoculation area, or the low proportion of the field that was sampled compared to 2017. Field 6 and 7, which were infested at a rate of one larva per 0.6 row-meter, had several positive samples extending to the 61.0 m sample zone by 3 DAI. Field 6 was infested with 283 infected larvae, and was positive for HearNPV in all six of the samples at 61.0 m, two out of five at 30.5 m, two out of three at 7.6 m, and one out of three in the inoculation area. Field 7 was infested with 410 infected larvae, and by 3 DAI there was one out of six samples positive at 61.0 m, three out of five at 30.5 m, and two out of three at 15.2 m. After the first sample date, only two samples were positive for HearNPV, and both

Table 5. All arthropods confirmed with PCR as voluntary carriers of HearNPV 3 DAI

Arthropods	Sample size (*n*)	No. positive for HearNPV						
		Distance (m)						
		0	7.6	15.2	30.5	61.0	Total	% Carriers
Lygus lineolaris	13	1	1	0	1	3	6	46.2
Diptera (Calliphoridae, Sarcophagidae, and Tephritidae)	18	0	0	0	0	5	5	27.8
Araneae	19	0	0	1	1	3	5	26.3
Hymenoptera[a] (Vespidae and Sphecidae)	3	0	0	0	0	3	3	100
Rivellia quadrifasciata	6	1	0	0	1	2	4	66.7
Ceresa festina	20	0	0	0	0	2	2	10.0
Geocoris spp.	7	0	0	0	0	2	2	28.6
Diaprotica undecimpunctata	10	0	0	0	0	2	2	20.0
Hypena scabra	22	0	0	1	1	0	2	9.1
Colaspis brunnea	19	0	1	0	0	0	1	5.3
Chinavia hilare	8	1	0	0	0	0	1	12.5
Cicindelinae	1	0	0	0	1	0	1	100
Chrysopidae[a]	2	0	0	1	0	0	1	50.0
Total	148	3	2	3	5	24	35	

[a]Denotes association with positive PCR samples by using Fisher's exact test ($\alpha = 0.05$).

Table 6. Arthropod families implemented as carriers, either voluntary or involuntary, of HearNPV and whether said carrier was confirmed through PCR

Order	Family	Species	Voluntary or Involuntary	Confirmed (PCR)
Diptera	Ulidiidae	*Rivellia quadrifasciata*	Voluntary	Yes
	Sarcophagidae		Voluntary	Yes
	Calliphoridae		Voluntary	Yes
	Tephritidae		Voluntary	Yes
	Tachinidae		Voluntary	No
	Milichiidae		Voluntary	No
Hemiptera	Miridae	*Lygus lineolaris*	Voluntary	Yes
	Pentatomidae	*Chinavia hilare*	Voluntary	Yes
	Geocoridae[a]	*Geocoris* spp.	Voluntary	Yes
	Reduviidae[a]		Voluntary	No
	Nabidae[a]		Voluntary	No
	Membracidae	*Ceresa festina*	Both	Yes
Hymenoptera	Vespidae	*Polistes* spp.	Voluntary	Yes
	Sphecidae		Voluntary	Yes
	Formicidae		Voluntary	No
Coleoptera	Carabidae	Cicindelinae	Voluntary	Yes
		Lebia spp.	Voluntary	No
	Coccinellidae[a]	*Coleomegilla maculata*	Voluntary	No
	Chrysomelidae	*Cerotoma trifurcata*	Involuntary	Yes
		Colaspis brunnea	Both	Yes
		Diabrotica undecimpunctata	Both	Yes
Neuroptera	Chrysopidae		Voluntary	Yes
Orthoptera	Acrididae		Involuntary	Yes
	Tettigoniidae		Both	Yes
Lepidoptera	Noctuidae	*Spodoptera ornithogalli*	Involuntary	Yes
	Erebidae	*Hypena scabra*	Both	Yes
Araneae[a]			Both	Yes

[a]Denotes a previously identified carrier of HearNPV.

were in field 7. One sample was positive from the inoculation area 10 DAI, and one was positive at the 7.6 m sample zone 14 DAI. We determined voluntary carriers, those that become carriers by contacting infected larvae, are capable of disseminating HearNPV 61.0 m, possibly further. However, voluntary carriers do not appear to be a viable method of viral residual presence due to only two samples being positive after 3 d. Therefore, although the data suggest these voluntary carriers are important in horizontal transmission potential through viral dissemination, they are not capable of sustaining HearNPV presence when *H. zea* larvae are not present.

Horizontal Transmission Potential of HearNPV

This study revealed regardless of how HearNPV was applied, as a bioinsecticide or as infected larvae, it was capable of disseminating up to 61.0 m within 3 d. This spread was not impacted by the presence of a host population, and could therefore potentially be contributed

to carriers, both involuntary and voluntary. However, residual presence of HearNPV seemed to be directly related to larval host populations. Fields with sustained *H. zea* had sustained HearNPV presence, and when *H. zea* were not present, HearNPV disappeared from the sampled area in a few days. This further emphasizes the importance of the host when evaluating horizontal transmission potential as proposed by Fuxa and Tanada (1987).

Previous research identified seven carriers of HearNPV, six predators and one parasitoid, which were ultimately determined to be insignificant in contributing to horizontal transmission (Young and Yearian 1990a,b). Our study identified 10 groups of arthropods confirmed with PCR as involuntary carriers, and 22 groups identified with PCR and observations as voluntary carriers (Table 6). Of these, the majority were not predaceous or parasitic. From these data we conclude that the arthropod complex is instrumental in disseminating HearNPV within a field, resulting in potential horizontal transmission. Also, predators and parasitoids are not the only arthropods carrying HearNPV and are possibly not the most important carriers. Future research could allow for a better understanding of these nonpredaceous, nonparasitic arthropods and the effect they have on potential horizontal transmission. By understanding the underlying biological factors, predicting the probability of an epizootic could be within reach.

Acknowledgments

The authors would like to thank AgBiTech LLC and the University of Arkansas Department of Entomology for supporting this work. They would also like to thank AgBiTech LLC for providing the virus used and for providing the primer sequences specific to HearNPV. The authors would also like to thank all the program associates and summer workers involved in collecting the samples utilized in this study.

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