Relationship Between Invasive Brown Marmorated Stink Bug (*Halyomorpha halys*) and Fumonisin Contamination of Field Corn in the Mid-Atlantic U.S.

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Abstract

Brown marmorated stink bug (*Halyomorpha halys* Stål) is an invasive agricultural pest that causes severe damage to many crops. To determine potential associations between *H. halys* feeding damage, *Fusarium* infection, and mycotoxin contamination in field corn, a field survey was conducted in eight counties in Virginia. Results indicated an association between *H. halys* feeding damage and fumonisin contamination. Subsequent field experiments in Delaware, Maryland, and Virginia examined the ability of *H. halys* to increase *Fusarium verticillioides* (Sacc.) Nirenberg infection and fumonisin concentrations in corn. At the milk stage, *H. halys* (0 or 4 adults) and *Fusarium* (with or without *F. verticillioides*

inoculum) treatments were applied to bagged ears in a two by two factorial randomized complete block design with 12 replicates. *H. halys* treatments increased levels of feeding damage ($P < 0.0001$) and *Fusarium* infection ($P = 0.0380$). Interaction between *H. halys* and *Fusarium* treatments influenced severity of infection ($P = 0.0018$) and fumonisin concentrations ($P = 0.0360$). Results suggest *H. halys* has the ability to increase both *Fusarium* infection and fumonisin concentrations in field corn. Further studies are needed to understand mechanisms by which *H. halys* increases fumonisin and to develop management strategies to mitigate impacts of *H. halys* on field corn in the region.

The brown marmorated stink bug, *Halyomorpha halys* Stål (*H. halys*), is an invasive insect pest that originated from eastern Asia. *H. halys* was first reported in the United States from Allentown, PA, in the late 1990s (Hoebeke and Carter 2003), and the insect quickly spread throughout Pennsylvania and into New Jersey, northern Delaware, Maryland, and Virginia (Hamilton 2009). Since then, *H. halys* has become a key pest in annual and perennial crops in the mid-Atlantic region and has also appeared in the Pacific Northwest (Lee 2015). *H. halys* is polyphagous and attacks over 300 economically important plant hosts, including field corn (*Zea mays* L.) (Leskey et al. 2012a,b; Nielsen and Hamilton 2009; Zhu et al. 2012). In mid-Atlantic field corn, peak *H. halys* pressure occurs during blister, milk, and dough stages (R2-R4) (Venugopal et al. 2015), when *H. halys* feed on developing kernels and reduce kernel quality by discoloring and shrinking kernels (Cissel et al. 2015; Hedstrom et al. 2014; Rice et al. 2014; Venugopal et al. 2014). This feeding damage occurs primarily on field edges, where up to 8% of kernels are damaged (Venugopal et al. 2014). Economic thresholds have not been developed for corn, and there have not been reports of stink bug feeding alone impacting marketable grain quality. However, the economic significance of *H. halys* damage could potentially be more severe if *H. halys* feeding increases incidence of fungal infections and mycotoxin contamination.

Insect feeding injury may facilitate ear invasion by opportunistic pathogens and may also induce stress that predisposes ears to diseases such as *Fusarium* ear rot (Avantaggiato et al. 2002; Munkvold 2003; Payne 1999; Smeltzer 1959). Ear rot, caused by *Fusarium* spp. including *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsush.) Nirenberg, and *F. graminearum* Schwabe, is common in the mid-Atlantic region and contributes to poor quality and mycotoxin contamination of corn grain (Stumpf et al. 2013). The FDA has advisory guidance for the *Fusarium* toxins fumonisin (mainly by *F. verticillioides* and *F. proliferatum*, <2 ppm to 100 ppm, depending on the

intended use) and deoxynivalenol (mainly by *F. graminearum*, 1 ppm to 5 ppm) in food and feed. There are no advisory guidelines for zearalenone, another toxin produced by certain *Fusarium* spp., but it is frequently associated with the presence of other mycotoxins and causes reproductive problems in livestock (El-Nezami et al. 2002; Kurtz and Mirocha 1978). The only mycotoxin for which the FDA has established regulatory levels in corn is aflatoxin (produced by *Aspergillus flavus* Link); action levels in corn range from 20 ppb (human consumption) to 300 ppb (feed for mature beef cattle). *A. flavus* infection and associated aflatoxin contamination, which are favored by hot, dry conditions, are less common in the mid-Atlantic but can be a significant problem in certain years (H. Mehl, unpublished data). Corn contaminated with mycotoxins near or above regulatory or advisory levels can result in poor prices or rejection at grain elevators, resulting in an economic loss for growers (Mitchell et al. 2016; Vardon et al. 2003).

Although the association of insects and mycotoxin contamination has been documented for a wide range of insect orders and species (Dowd 1998), the extent to which *H. halys* contributes to fungal infection and mycotoxin contamination has not been adequately studied. Previous research in the southeastern United States demonstrated an association between native brown stink bug (*Euschistus servus*) feeding and preharvest aflatoxin contamination in corn (Ni et al. 2010, 2011). However, in the mid-Atlantic, native stink bug populations have not typically been at high enough numbers to cause significant damage. Mass *H. halys* invasion of corn fields in the region may cause greater levels of feeding injury and potentially lead to increases in mycotoxin contamination.

Prior and current research on *H. halys* has focused on understanding the biology of the insect, the damage caused by *H. halys* to a variety of crops, and the most effective strategies for mitigating damage and crop losses from this invasive pest (Cissel et al. 2015; Hoebeke and Carter 2003; Leskey et al. 2012a,b; Rice et al. 2014; Venugopal et al. 2014). However, the extent to which *H. halys* impacts mycotoxin contamination levels in field corn has not been determined. Thus, the objectives of this research in field corn were to: 1) determine if there is a relationship between *H. halys* infestation, feeding injury, and the presence of mycotoxin contamination, and 2) examine the ability of *H. halys* to increase *Fusarium* infection and fumonisin contamination.

Materials and Methods

Field survey. In July 2013, when the corn crop was at or near the milk stage, high numbers of *H. halys* (10 or more per ear) were

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observed along the edges of corn fields throughout central Virginia. In mid-September just prior to harvest, corn ears were collected from fields where *H. halys* infestations had been documented. A total of eight fields were sampled in Albemarle, Powhatan, Orange, Madison, Culpeper, Frederick, King George, and Spotsylvania counties. At each field, two to four corn ears were collected from three different locations along the field edge where high levels of *H. halys* feeding damage was observed earlier in the growing season. Since *H. halys* infestations and signs of feeding damage were confined primarily to field edges, a second set of two to four ears were collected 12 to 20 rows into the field (straight in from each edge location) so damaged and undamaged ears could be compared. Ears were hand-shelled with a metal corn sheller (Decker Manufacturing, Keokuk, IA) and kernels were dried in paper bags at 45°C for 72 h.

Assessment of kernel damage. Shelled kernels were thoroughly mixed by hand, and damage was quantified from a 70 g subsample. Kernels were visually inspected to determine the percentage of kernels with evidence of stink bug feeding damage, which includes a puncture scar surrounded by a discolored cloudy marking, and sunken, shrunk, and collapsed kernels as described previously (Cissel et al. 2015; Hamilton and Shearer 2003; Venugopal et al. 2014).

Fungal infection. Frequency of fungal infection was determined from 20 randomly selected kernels from each sample. Kernels were surface disinfested with 10% commercial bleach for 2 min, air dried, and plated (5/plate) on 1/4 strength potato dextrose agar (PDA, 9.75 g/liter) (Fisher Scientific, Ottawa, Canada) amended with 100 mg/liter chloramphenicol and 100 mg/liter chlortetracycline. Plates were incubated at room temperature (~25°C) for 5 to 7 days. The number of kernels with fungal growth was quantified, and fungi were identified to genus and in some cases species based on spore and culture morphology (Barnett and Hunter 1998; Leslie and Summerell 2006).

Single-spore purification of isolates. *Fusarium* isolates were grown on full strength PDA (39 g/liter) for 5 days. A piece of the growing *Fusarium* culture was picked with a sterile toothpick and gently streaked across the surface of 2% water agar. Following incubation (25°C, 16 h), water agar plates were inspected under a dissecting microscope (10×) to identify germinating conidia. A sterile toothpick was used to cut out and transfer a single germinating conidium to full strength PDA. Single-spore isolates were grown at 25°C for 4 to 7 days to obtain a pure culture.

Filter paper archival cultures. Sterilized Whatman #1 qualitative filter papers of 7 cm diameter were cut into two and each half placed on full strength PDA approximately 3 mm apart. Single-spore purified *Fusarium* cultures were then transferred to the center of the plate and allowed to grow at 25°C for 5 days. Colonized filter paper was aseptically transferred to sterile empty Petri dishes and oven dried at 45°C for 72 h. Sterilized scissors were used to cut one of the dried filter papers into pieces (about 5 × 5 mm) and placed in a sterile 7.62 × 11.43 cm envelope (Uline, Atlanta, GA) and kept at 4°C. A piece of the cut filter paper was used to regenerate a copy of the isolate when needed. The other half was kept at -20°C for long-term storage.

Molecular identification of *Fusarium* spp. Seven representative isolates with different culture and spore morphologies were sequenced to confirm *Fusarium* species identification. Fungal DNA was isolated using the FastDNA SPIN kit (MP Biomedicals, Solon, OH). Samples were homogenized in a Mini-BeadBeater 24 (Bartlesville, OH) for 40 s at a speed of 3,000 rpm. Subsequent steps were performed according to the manufacturer's protocol. DNA was eluted with a final volume of 200 µl of ddH₂O. The translation elongation factor (TEF1-α) gene was amplified using HotStart PCR Pre-Mix (Bioneer, Inc., Alameda, CA) with 0.2 µM of the forward primer and reverse primers (EF1 and EF2, O'Donnell et al. 1998) and 10 ng template DNA in a total volume of 20 µl. PCR was performed using a Bio-Rad T 100 thermocycler with the following conditions: an initial denaturing step at 94°C for 5 min followed by 35 cycles of 94°C for 40 s, 53°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 5 min. The amplicons were run on a 1.5% agarose gel with 1× Tris/Borate/EDTA (TBE) as buffer and gel red as the DNA intercalating fluorescent dye. Amplicons were visualized under UV

light by using molecular imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA) to confirm the amplicon size of ~700 bp. Five microliters of the amplicon was purified using ExoSap-IT kit (Affymetrix Inc., Santa Clara, CA) following the manufacturer's instructions. The purified amplicons were submitted to the Virginia Biotechnology Institute (VBI), Virginia Tech, Blacksburg, VA, for sequencing in both directions. A consensus sequence was generated by aligning the forward and reverse sequences using Geneious software version 8.1, and a BLAST search of the National Center for Biotechnology Information (NCBI) databases was performed to determine the species. Sequences were deposited in GenBank (accession nos. MH496628–MH496634).

Mycotoxin extraction and quantification. Neogen's Reveal Q+ kits (Neogen Corporation, Lansing, MI) were used to quantify fumonisin, deoxynivalenol, zearalenone, and aflatoxin from each corn sample. Kernels were pulverized using a Bunn-O-matic gourmet coffee grinder (Bunn-O-matic Corporation, Springfield, IL). For extraction of fumonisin, zearalenone, and aflatoxin, 50 ml of 65% ethanol was added to 10 g of each sample and the mixture was vigorously shaken in a floor model shaking incubator (VWR Corporate, Suwanee, GA) for 3 min. For extraction of deoxynivalenol, 100 ml distilled water was added to 10 g of sample and the sample was mixed as described above. Toxins were then extracted and quantified according to the manufacturer's protocol using Reveal Q+ test strips and the Accuscan Pro instrument. The ranges of detection were 0.3 to 6 ppm for fumonisin, 50 to 1,200 ppb for zearalenone, 2 to 150 ppb for aflatoxin, and 0.3 to 6 ppm for deoxynivalenol. If samples exceeded the upper limit of detection, they were diluted 1:10 in the sample diluent provided with the kit and requantified using a new test strip.

Assessment of *H. halys* impacts on fungal infection and mycotoxin contamination. Based on the 2013 field survey, it was determined that *H. halys* infestations were most frequently associated with *F. verticillioides* ear rot and fumonisin contamination. An experiment including *H. halys* and *F. verticillioides* treatments was conducted and replicated three times to demonstrate reproducibility. Experiments were replicated across three field locations (Delaware, Maryland, and Virginia) in 2015. Since this was a "proof of concept" study to determine if *H. halys* is able to increase fungal infection and mycotoxins rather than a study to look at the magnitude of the effect across locations/environments, it was conducted in a single year with the different locations as independent, replicated experiments. Bt corn hybrid N68B-3111 treated with Cruiser Maxx (Syngenta U.S., active ingredients: thiamethoxam insecticide, mefenoxam fungicide, and fludioxinil fungicide) was planted at a seeding rate of approximately 30,000 seeds per acre and managed according to each location's cooperative extension service guidelines. For the experiment, four rows spaced 30 inches apart were utilized for all locations. Records of temperature, rainfall, and relative humidity for Virginia, Maryland, and Delaware were obtained from the Peanut-Cotton InfoNet, Central Maryland Research and Education Center (CMREC) Beltsville Facility, and Delaware Environmental Observing System (DEOS), respectively. Descriptions of experimental sites and dates of planting, application of treatments, and harvest are shown in Table 1.

Source of *H. halys* colonies. *H. halys* used for the experiment in the Virginia location were collected from forest edges in Blacksburg, VA, just prior to initiation of the experiment. *H. halys* cultures for the Delaware location were obtained from Phillip Alampi, Beneficial Insect Rearing Laboratory, New Jersey Department of Agriculture, Division of Plant Industry, Trenton, NJ. *H. halys* for the Maryland location were collected from soybean fields in Clarksville, MD, and held in the laboratory under typical colony conditions (as described in Taylor et al. 2017) until field deployment 10 days later.

Fungal inoculum. *F. verticillioides* isolated from fumonisin-contaminated corn collected from Spotsylvania County, Virginia, during the 2013 field survey was used for inoculum in all three experiments. *F. verticillioides* suspension was made by growing pure cultures on full strength PDA for 5 days at 25°C and scraping the culture (mostly conidia) into 500 ml of sterile, purified water. The conidial

suspension was then vigorously shaken to mix. The concentration of the conidial suspension was determined by using a hemocytometer and a final concentration was adjusted to 1×10^6 conidia/ml.

Experimental design and treatment application. The experiment consisted of a factorial arrangement of treatments in a randomized complete block design. The main factors were *H. halys* treatments (zero or four adult *H. halys* per ear) and *Fusarium* treatments (water or conidial suspension of *F. verticillioides*). Thus, the four treatment group combinations were untreated control, *H. halys* only, *F. verticillioides* only, and *H. halys* plus *F. verticillioides*. At tasseling, 12 ears of uniform size were selected from each of the four rows for application of treatments. Selected ears were covered with mesh bags (28 by 29 cm, 3.8-liter mesh elastic top bag strainer, Trimaco, Morrisville, NC) to prevent insect feeding. At the R3 (milk) stage, treatments were randomly applied to the selected individual ears of plants in four rows of corn. Four milliliters (10 sprays) of *F. verticillioides* conidial suspension or water were applied to the surface of the developing ear using a handheld spray bottle. For *H. halys* infestation treatments, four adult *H. halys* were applied to bagged ears. Each treatment was replicated three times in each row resulting in 12 experimental units (ears) per row and 48 experimental units per experiment. After 7 days, *H. halys* were removed but ears remained covered with mesh bags until harvest. At maturity, the corn ears were hand harvested and dried at 45°C for 72 h to reduce the moisture level to approximately 15%. Signs of *H. halys* feeding damage, kernel infection, and fumonisin concentrations were determined for each sample as described above.

Quantification of *F. verticillioides* DNA (qPCR). To estimate the total quantity of *F. verticillioides* colonizing the kernels, real-time quantitative PCR (qPCR) was conducted using previously developed *F. verticillioides*-specific primers (Mulè et al. 2004). Two hundred milligrams of ground corn was weighed into a lysing matrix A tube, and DNA was isolated following FastDNA SPIN kit (MP Biomedicals, Solon, OH) protocol with the modifications indicated above. Prior to running the samples, a SYBR green assay was validated and optimized to ensure accuracy and reproducibility of the quantification of samples. The reaction mixture for the qPCR was run under temperature gradient from 55 to 65°C to determine the optimum annealing temperature. The melt curve generated from the qPCR reaction analysis was evaluated to confirm primer specificity and fidelity. Real-time qPCR reaction mixtures included 5 µl of SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 3 µl of DNA template, and 1 µl each of the 250 nM forward and reverse *F. verticillioides* specific primers (Mulè et al. 2004) in a total volume of 10 µl. Each sample was analyzed in triplicate. Each run included a standard curve in triplicate that consisted of 10-fold dilutions of *F. verticillioides* DNA from 6 to 6×10^{-5} ng per reaction. Reactions were performed on a Bio-Rad Laboratories CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation of 98°C for 3 min followed by 40 cycles of 98°C for 15 s, 62°C for 2 min, and a plate read after each cycle. A melt curve was run from 65 to 95°C with 0.5°C increments for 5 s and followed by a plate read. Based on the standard curve, quantities of *F. verticillioides* DNA in each sample were calculated by the Bio-Rad CFX Connect Real-Time PCR Detection System software.

Statistical analyses. In order to meet the assumptions of normality and homogeneity of variance for an analysis of variance, percentage

data and concentration data were arcsine and log transformed, respectively. For the field survey, field location (county) and sample location (edge versus interior of field) were included as variables in the analysis. The effects of field location and sample location on percent kernel infection and mycotoxin concentrations were assessed with an analysis of variance.

For the analysis of the field experiment, individual ears served as the experimental units. The experiment was analyzed as a full factorial with *H. halys* treatment, *Fusarium* treatment, and experiment (location) as dependent variables in the analysis of variance. Comparison of least square means was conducted using the LSMeans Tukey's HSD. Values were considered significantly different at $P < 0.05$ and JMP Pro version 13.0.0 (SAS Institute Inc., Cary, NC) was used for the analysis of all data in both experiments. Presented values are back-transformed means.

Results

Field survey. Signs of stink bug feeding injury ranged from 0.6 to 40.1% of the kernels on ears collected from eight corn fields that were infested with *H. halys* in 2013. Ears collected from field edges had higher levels of feeding injury compared with ears collected from field interiors (22.5 versus 2.0% of kernels, $P < 0.0001$). The fungal genus most frequently isolated from kernels was *Fusarium* (Table 2). Based on morphology, most of the isolates resembled *F. verticillioides*, a causal agent of fumonisin contamination in corn. Based on a BLAST search of seven sequenced isolates representing the different *Fusarium* morphotypes, four were confirmed

Table 2. Variation in frequencies of fungal infection and concentrations of mycotoxins in kernels collected from field edges and interiors at eight Virginia corn fields infested with *Halysomorpha halys* in 2013^x

Variable ^y	Mean		P-value ^z		
	Field edge	Field interior	Location	Edge versus interior	Interaction
<i>Fusarium</i> , %	31	18	0.06	0.02	0.98
<i>A. flavus</i> , %	0.4	0	0.28	0.07	0.28
<i>A. niger</i> , %	3	2	0.49	0.72	0.21
<i>Penicillium</i> , %	7	9	0.16	0.43	0.50
Other fungi, %	4	5	0.50	0.86	0.68
Total infection, %	44	32	0.04	0.08	0.81
Fumonisin, ppm	13.3	1.9	0.0005	<0.0001	0.28
Zearalenone, ppb	99	81	<0.0001	0.06	0.11
Deoxynivalenol, ppm	0.4	0.5	0.0012	0.21	0.90
Aflatoxin, ppb	0.9	0.9	0.0002	0.25	0.47

^x Fields were selected where high levels of *H. halys* feeding were observed earlier in the season. For each field, two to four corn ears were collected from three sampling locations each along the field edge and interior (12 to 20 rows into the field from each edge location).

^y Both fungal infection and mycotoxin concentrations were assessed for each sample. Infection with fungal pathogens was assessed by plating 20 randomly selected kernels on 1/4 strength potato dextrose agar. After 1 week, the number of kernels with fungal growth were counted and fungal species were identified based on morphological characteristics. Mycotoxin concentrations were quantified from ground kernels with Neogen Reveal Q+ kits.

^z For each measured variable (% infection or mycotoxin concentrations), the effects of field location and location within the field (edge versus interior) were assessed with an analysis of variance.

Table 1. Summary of experimental sites, cropping history, and dates of field corn planting, treatment application, and harvest in Delaware, Maryland, and Virginia, where experiments were conducted to assess the impacts of *Halysomorpha halys* feeding on *Fusarium verticillioides* infection and fumonisin contamination in 2015

Experiment (location)	Experimental site	Previous crop	Planting date	Treatment date ^z	Harvest date
DE	Newark Agriculture Research Station	Corn	1 Jun	17 Aug	21 Sep
MD	Central Maryland Research and Education Center (Beltsville)	Vegetables	29 Jun	11 Sep	26 Oct
VA	Tidewater Agricultural Research and Extension Center (Suffolk)	Cotton	11 Jun	3 Sep	7 Oct

^z *H. halys* and *F. verticillioides* treatments were applied at the milk (R3) stage of kernel development in the crop.

as *F. verticillioides*. The other three isolates were *F. proliferatum* (two isolates) and *F. incarnatum* (Roberge) Sacc. (one isolate). Other isolated fungi included *A. flavus*, *A. niger* Tiegh., and *Penicillium* spp. A greater proportion of the kernels collected from field edges were infected with *Fusarium* compared with kernels collected from field interiors ($P = 0.02$). The proportion of corn kernels infected with the other fungi did not differ between the edge and middle of fields (Table 2).

Fumonisin, zearalenone, deoxynivalenol, and aflatoxin were all detected from corn kernels, but only fumonisin exceeded FDA advisory levels (Table 2). Concentrations of all four mycotoxins varied among locations, but fumonisin was consistently higher in corn collected from field edges than in corn collected from field interiors. Fumonisin concentrations were positively correlated with the proportion of kernels infected with *Fusarium* spp. ($r^2 = 0.33$, $P < 0.0001$) and the proportion of kernels with signs of stink bug feeding injury ($r^2 = 0.26$, $P = 0.0004$). Subsamples of injured kernels separated from uninjured kernels were also analyzed and in some cases had over 100 ppm fumonisin (data not shown).

Assessment of *H. halys* impacts on fungal infection and mycotoxin contamination. Maximum and minimum temperatures between planting and the application of treatments at the milk (R3) stage of the corn crop were similar among experimental locations (Table 3). However, rainfall varied among experiments with the MD location receiving less than half the rainfall recorded at DE and VA locations. Between application of treatments (R3) and harvest, temperatures were cooler at the MD location compared with DE and VA locations, and the DE location received considerably less rainfall compared with the other two locations. Overall, the warm temperatures and consistent rainfall at the VA location were more favorable for fungal infection compared with the DE and MD locations.

Following harvest, signs of *H. halys* feeding injury (discolored, sunken, collapsed, and aborted kernels) ranged from 0% of the kernels from the untreated ears to a maximum of 27% from the *H. halys* treated ears. The percentage of kernels on harvested ears with signs of *H. halys* feeding injury varied among *H. halys* treatments ($P < 0.0001$) and experiments ($P < 0.0001$) but did not vary with *Fusarium* inoculation treatment ($P = 0.98$). There were no treatment interactions or treatment by experiment interactions ($P > 0.05$), so main effects were analyzed. Percent injured kernels was higher in the VA experiment compared with the experiments at the other two locations, but overall, treatments that included *H. halys* infestation had significantly more injury compared with treatments without *H. halys* (Table 4).

Frequency of *Fusarium* infection varied among *H. halys* treatment ($P = 0.038$), *Fusarium* treatment ($P = 0.019$), and experiments ($P < 0.0001$). Over 40% of the kernels at the VA location were infected with *Fusarium* whereas only 11.7% of kernels were infected at the MD location. This was likely due to warm, wet temperatures in VA and cool temperatures in MD following application of the fungal inoculum (Table 3). However, despite variation in overall levels of

infection across experiments, there were no treatment by experiment interactions ($P > 0.05$). Across all three experiments, ears infested with *H. halys* or inoculated with *Fusarium* had higher frequencies of *Fusarium* infection compared with nontreated ears (Table 4).

Infection severity based on quantified *F. verticillioides* DNA in the kernels differed among *H. halys* treatment ($P = 0.022$), *Fusarium* treatment ($P < 0.0001$), and experiment ($P < 0.0001$), and there was a significant *H. halys* by *Fusarium* interaction ($P = 0.0018$). However, treatment effects were similar across locations (treatment by experiment interactions, $P > 0.05$). *F. verticillioides* DNA concentration was highest in the VA samples (mean = 9.8 ng *F. verticillioides* DNA/g corn), whereas DE and MD samples had concentrations 0.03 and 0.002 ng/g, respectively. The highest levels of infection were detected in ears that were both infested with *H. halys* and inoculated with *F. verticillioides*; levels of infection were similar among untreated ears and those with either a *H. halys* or *Fusarium* treatment alone (Fig. 1).

Fumonisin concentrations in harvested grain varied among locations ($P = 0.035$). For the DE experiment, the 22% of samples with detectable fumonisin ranged from 0.3 to 34 ppm (mean = 8 ppm), with an overall mean of 0.54 ppm for all samples. For the VA experiment, 25% of samples with detectable fumonisin ranged from 0.4 to 17 ppm (mean = 4.1 ppm) with an overall mean of 0.50 ppm for all samples. The MD experiment had the lowest frequency and concentration of detectable fumonisin with 4% of samples with detectable fumonisin ranging from 3 to 12 ppm (mean = 7.5) with an overall mean of 1.1 ppm. There was an interaction between *H. halys* and *Fusarium* treatments ($P = 0.036$) and fumonisin concentrations were highest when both *H. halys* and *Fusarium* treatments were applied (Fig. 1). *H. halys* or *Fusarium* treatment alone did not increase fumonisin concentrations in the grain.

Discussion

In this study, the relationship between *H. halys*, *Fusarium* infection, and mycotoxin contamination was characterized, and an interaction between *H. halys* feeding and fumonisin contamination was identified. This suggests that invasive *H. halys* not only directly injures the crop through feeding but may also indirectly increase the impacts of mycotoxigenic fungi. This relationship thereby increases *H. halys* damage potential in field corn, and this may have implications for corn pest management in the mid-Atlantic as invasive *H. halys* spreads throughout corn production areas.

H. halys can cause significant economic damage in orchard crops (e.g., apples, peaches) and fresh vegetables (e.g., tomato, pepper, sweet corn) where direct damage to the marketable portion of the crop reduces quality and marketable yield (Kuhar et al. 2012; Leskey et al. 2012a; Rice et al. 2014). In field corn, other native species of stink bugs can cause feeding injury but typically do not cause economic damage (Clower 1958; Ni et al. 2010). In contrast, the higher population densities of *H. halys* found in field corn and other crops

Table 3. Mean daily maximum and minimum temperature and total rainfall between planting and application of *Halyomorpha halys* and *Fusarium verticillioides* treatments to corn ears and between treatment application and harvest at three locations where experiments were conducted to assess the impacts of stink bug feeding on fungal infection and fumonisin contamination^y

Experiment (location)	Planting to R3 ^z			R3 ^z to harvest		
	Temperature (°C)		Rainfall (mm)	Temperature (°C)		Rainfall (mm)
	Max	Min		Max	Min	
DE	28.6	18.7	495	28.9	16.7	72
MD	30.2	17.8	140	21.5	9.1	236
VA	32.3	20.3	354	27.7	17.7	201

^y Planting, treatment, and harvest dates are in Table 1.

^z Treatments were applied at the milk (R3) stage of the corn crop.

Table 4. Main effects of *Halyomorpha halys* infestation, *Fusarium verticillioides* inoculation, and experiment on frequencies of stink bug feeding injury and *Fusarium* infection in corn

Treatment factor	Level	Feeding injury, % ^{x,y}	<i>Fusarium</i> infection, % ^{x,z}
<i>H. halys</i>	Yes	4.7 a	32.1 a
	No	1.7 b	23.8 b
<i>F. verticillioides</i>	Yes	3.1 a	32.2 a
	No	3.0 a	23.7 b
Experiment (location)	DE	2.4 b	30.8 b
	MD	2.1 b	11.7 c
	VA	5.0 a	44.5 a

^x Percentage data were arcsine transformed prior to analysis of variance, and detransformed means are presented. Means followed by different letters are significantly different according to Tukey's HSD.

^y Percent of harvested kernels with signs of stink bug feeding injury.

^z Percent of harvested kernels infected with *Fusarium* based on plating 20 kernels per corn ear sample on 1/4 strength potato dextrose agar.

result in a greater potential for economic damage. *H. halys* feed primarily on field edges (Venugopal et al. 2014), which localizes damage, but application of effective chemical controls is difficult in field corn that has passed the tasseling stage (Rice et al. 2014). Indeed, the poorly understood economic importance and lack of management strategies for *H. halys* in field corn have hindered the development of an economic threshold.

This study identifies and quantifies potential economic impacts of *H. halys* resulting from mycotoxin contamination. Both the field survey and the field experiment demonstrated the ability of *H. halys* to increase *Fusarium* infection and fumonisin contamination. In the field survey, the observation of increased *H. halys* population numbers and associated injury occurring at the edge of corn fields compared with the middle is consistent with other studies focused on apple, peach, soybean, and ornamentals (Leskey et al. 2012a). Therefore, comparing samples collected from the edge and the middle (12 to 20 rows from the edge) was a useful approach to characterizing the impact of *H. halys* within the same surveyed field. A shortcoming of the field survey was that it was not possible to distinguish between kernel feeding injury caused by *H. halys* and that of native stink bugs and other insects. However, high numbers of *H. halys* were observed during the growing season in the sampled fields, and the injury assessed on the kernels resembled previous descriptions of *H. halys* feeding. Also, since *H. halys* infestation was greatest at the edges compared with interior portions of fields, “edge effect” factors may have contributed to increased levels of fungal infection and mycotoxin contamination. Plants grown at the edges of agriculture fields often experience harsh treatments such as excess chemical deposit, drought, and poor soil nutrient leading to poor plant health and stress. Poor plant health and stress may reduce their capacity to resist pathogen invasion and subsequent mycotoxin contamination (Miller 2001). Again, the survey was conducted at multiple locations with variable levels of *H. halys* infestation and different environmental conditions leading to variable amount of damage, fungal infections, and mycotoxin contamination. Nevertheless, the pattern of high damage, high infection, and high fumonisin concentrations was associated with *H. halys* infestation across all locations.

In the experiment conducted to assess the impacts of *H. halys* on *F. verticillioides* infection and fumonisin concentrations, relatively low levels of *H. halys* feeding injury, *Fusarium* infection, and fumonisin concentrations were detected compared with the field survey. This may have been influenced by environmental conditions at the time of the experiments. However, a lack of treatment by experimental location interactions indicates treatment effects were similar across experiments. Although injury was low, only *H. halys* treatment significantly affected injury measurements, which indicates observed damage was primarily due to *H. halys* feeding and not fungal infection or other insect damage. Environmental conditions in Virginia were favorable for infection and *H. halys* feeding and the Virginia site exhibited relatively high injury, infection, and fumonisin concentrations. The lower injury, incidence and severity of infection, and fumonisin concentrations recorded in Maryland could be attributed to late planting, which caused the experiment to coincide with colder environmental conditions. It is possible that the low temperatures may have reduced *H. halys* feeding and also suppressed the growth and infection of *F. verticillioides* leading to low levels of injury, infection, and subsequent fumonisin concentration. The Delaware site experienced consistently favorable temperatures for *F. verticillioides* infection from treatment application to harvest, but the long period of drought during that time may have limited infection and induced drought stress in the corn. Warm (>27°C) and droughty conditions favor *Fusarium* ear rot development and subsequent fumonisin contamination (Miller 2001; Pascale et al. 1997; Shelby et al. 1994), which could explain the relatively high fumonisin concentrations in Delaware samples. *Fusarium* infection was detected in kernels from noninoculated ears, but this is not unexpected since *F. verticillioides* and other *Fusarium* spp. are common in the environment and *F. verticillioides* can survive as an endophyte without resulting in any symptoms (Bacon and Hinton 1996; Leslie and Summerell 2006; Yates et al. 1997). However, systemic infections appear to

contribute little to ear mold symptoms and fumonisin concentrations (Desjardins et al. 2002; Munkvold et al. 1997).

Even though this experiment demonstrates the ability of *H. halys* to increase *F. verticillioides* infection and fumonisin concentrations, it does not demonstrate possible mechanisms responsible for these effects. Possibly, the feeding damage served as a channel for invasion as demonstrated in other studies (Avantaggiato et al. 2002; Munkvold 2003; Payne 1999; Smeltzer 1959; Yates and Sparks 2008). Also, kernel infection through the inoculation of silk with spore suspension is possible (Munkvold et al. 1997; Reid et al. 1992) and that may in part explain why some ears without observable damage had high frequency of infection. Thus, it is possible that *H. halys* feeding injury is what facilitated infection and the resultant fumonisin contamination, and it is also likely that silk infection prior to treatment application may have contributed to the observed kernel infection.

Another possible mechanism for the increased infection could be that *H. halys* may be vectoring *Fusarium* spp. either externally or internally, thereby increasing the quantity of inoculum present during the time of feeding and facilitating infection and mycotoxin contamination. The vectoring capacity of *H. halys* has been reported in other studies. *H. halys* was found to be a vector for the phytoplasma that

Fig. 1. Impact of brown marmorated stink bug (BMSB) (*Halyomorpha halys*) and *Fusarium verticillioides* treatments on fungal infection severity (top) and fumonisin concentrations (bottom) in corn kernels. *H. halys* (with or without four adult *H. halys*) and *F. verticillioides* (with or without inoculation) treatments were applied to corn ears at the milk (R3) stage of kernel development in a factorial design. Following harvest, fungal infection was quantified by real-time PCR using *F. verticillioides*-specific primers, and fumonisin concentrations were determined using a Neogen Reveal Q+ kit. Data were log transformed prior to an analysis of variance, and there was a significant *H. halys* by *F. verticillioides* treatment interaction for both infection ($P = 0.0018$) and fumonisin ($P = 0.0360$). Means were compared using Tukey's HSD, and detransformed means are presented. Bars with different letters are significantly different from each other according to Tukey's HSD.

causes witches'-broom in the princess tree (*Paulownia tomentosa*) in Japan, Korea, and China (Bak et al. 1993; Hiruki 1999; Weintraub and Beanland 2006). The capacity of *H. halys* to phoretically carry infective *Colletotrichum* spores from diseased tomatoes to healthy ones has been demonstrated (Voshell et al. 2015).

Only fumonisin was associated with *H. halys* in this study, and other studies have reported a significant correlation between extent of insect damage and fumonisin levels in field corn (Dowd 2001; Munkvold et al. 1999). However, it is possible that environmental conditions during the growing season may have contributed to this association rather than the inability of *H. halys* to increase deoxynivalenol or aflatoxin contamination in corn. For example, *A. flavus* infection and associated aflatoxin contamination, which are favored by hot, dry, and drought conditions (Diener et al. 1987; Klich 1987), are less common in the mid-Atlantic but can be a significant problem in seasons with favorable weather conditions. Also, *F. verticillioides* sporulates greatly under humid and warm conditions of about 27°C (Rossi et al. 2009), which corresponds to typical environmental conditions in the mid-Atlantic during the summer months when corn is grown. Other studies have also shown that corn kernels colonized by *F. verticillioides* are less likely to be significantly colonized by other mycotoxin producers such as *F. graminearum*, *F. subglutinans*, and *Aspergillus* spp. (Marín et al. 1998a, b; Rheeder et al. 1990); hence, *F. verticillioides* infection would have the greatest frequency if it was first to colonize the ear.

H. halys is invasive and is likely to spread throughout the U.S. within a few years (Holtz and Kamminga 2010; Zhu et al. 2012); hence, it will continue to be a potential economic threat to a wide range of agricultural production systems in the United States. It is also a risk factor for fumonisin and perhaps other mycotoxin contamination in field corn, and mycotoxin concentrations may increase as a result of *H. halys* population increases and spread across the landscape. Risk of mycotoxin contamination associated with *H. halys* can be mitigated by better characterizing the relationship between *H. halys* and mycotoxin-producing *Fusarium* spp., including testing the ability of *H. halys* to vector *Fusarium* spp., quantifying overall impact of *H. halys* infestations on mycotoxin contamination especially at the edges of the field where their populations are frequently high. It may also be important to monitor mycotoxin contamination events over multiple sites and states where *H. halys* occurs and correlate that with insect and fungal control practiced by growers to determine best practices that ameliorates their impacts. During high *H. halys* infestation, it may be precautious for growers to practice selective harvest (Ni et al. 2011). Thus, growers should harvest the middle parts of their fields separate from the edges of their fields and assess both harvests separately for damage and mycotoxin contamination and only mix them when necessary. Although the Cry1 Ab protein in Bt corn dramatically reduced corn-borer damage leading to less *Fusarium* infection and fumonisin contamination (Betz et al. 2000; Hammond et al. 2004), *H. halys* is able to cause substantial damage, which may be a precursor for high *Fusarium* infection and fumonisin contamination. Therefore, the search for new natural fungal and insect resistance mechanisms and developing hybrids that are resistant to insect damage and fungal infection or produce enzymes that degrade fumonisin as it is produced would reduce mycotoxin incidence appreciably (Abbas et al. 2013; Cleveland et al. 2003). Also, because the *H. halys* adults are mobile and move in large numbers, they attack large numbers of plants at a time and cause extensive damage. Quarantine agencies and other players in the agriculture sector must be interested in the infestation dynamics of *H. halys* so that commodities coming from areas with high *H. halys* infestation would be critically scrutinized for mycotoxins since apart from feeding damage reducing quality, such commodities may have an additional risk of mycotoxin contamination.

In conclusion, the results suggest *H. halys* has the ability to increase both *Fusarium* infection and fumonisin concentrations in field corn. However, further studies are needed to understand mechanisms by which *H. halys* increases fumonisin, and possibly other mycotoxins. Studies about *H. halys* transmitting mycotoxin-producing fungi during feeding and how that contributes to the total mycotoxin

contamination should be conducted. Also, further studies have to be carried out to understand the population dynamics of *H. halys* and set population threshold in field corn that would serve as a critical control point. Additional studies should be carried out to estimate how much selective harvest would contribute to a reduced impact of mycotoxin contamination in *H. halys*-infested corn fields. Understanding of the mechanisms involved in the increased mycotoxin contamination in field corn would help in the formulation of management strategies to mitigate impacts of *H. halys* on field corn in the region.

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