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Entomopathogens infecting brown marmorated stink bugs before, during, and after overwintering

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The microsporidian, *Nosema maddoxi* Becnel, Solter, Hajek, Huang, Sanscrainte & Estep, infects brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), populations in North America and Asia and causes decreased fitness in infected insects. This host overwinters as adults, often in aggregations in sheltered locations, and variable levels of mortality occur over the winter. We investigated pathogen prevalence in *H. halys* adults before, during, and after overwintering. Population level studies resulted in detection of *N. maddoxi* in *H. halys* in 6 new US states, but no difference in levels of infection by *N. maddoxi* in autumn versus the following spring. *Halyomorpha halys* that self-aggregated for overwintering in shelters deployed in the field were maintained under simulated winter conditions (4°C) for 5 months during the 2021–2022 winter and early spring, resulting in 34.6 ± 4.8% mortality. Over the 2020–2021 and 2021–2022 winters, 13.4 ± 3.5% of surviving *H. halys* in shelters were infected with *N. maddoxi*, while *N. maddoxi* infections were found in 33.4 ± 10.8% of moribund and dead *H. halys* that accumulated in shelters. A second pathogen, *Colletotrichum fioriniae* Marcelino & Gouli, not previously reported from *H. halys*, was found among 46.7 ± 7.8% of the *H. halys* that died while overwintering, but levels of infection decreased after overwintering. These 2 pathogens occurred as co-infections in 11.1 ± 5.9% of the fungal-infected insects that died while overwintering. Increasing levels of *N. maddoxi* infection caused epizootics among *H. halys* reared in greenhouse cages after overwintering.

Key words: entomopathogens, *Halyomorpha halys*, overwintering, *Nosema maddoxi*, *Colletotrichum fioriniae*

Introduction

One overwintering strategy used by insects in temperate climates is for individuals to find sheltered locations in the autumn, within which they remain inactive and often in diapause until the following spring. Adults of some of these taxa often overwinter in aggregations (e.g., hemipterans, coleopterans, and lepidopterans) and this can enhance their fitness and survivorship by mitigating the effects of abiotic stressors, such as low temperatures and desiccation, during this period (Yoder and Smith 1997, Su et al. 2007, Murakami et al. 2019, Szejner-Sigal and Williams 2022). However, biotic stressors such as increased infection by pathogens that can negatively impact survival have also been reported for species that aggregate for overwintering. For example, significant levels of infection by the lethal fungal pathogen *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin have been documented in overwintering aggregations of the coccinellids, *Coccinella septempunctata* (L.) (Güven et al. 2015) and *Olla v-nigrum* (Mulsant) (Cottrell and Shapiro 2003).

In North America, the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål), has been a significant agricultural and nuisance pest since the late 2000s (Leskey and Nielsen 2018). Diapausing adults of *H. halys* overwinter from September–October to April–May (Bergh et al. 2017, Bergh and Quinn 2018), often in aggregations that typically occur in tight spaces within dry and dark locations (Toyama 2006, Toyama et al. 2011, Cullum et al. 2020) in both natural settings outdoors (Lee et al. 2014) and inside buildings (Hancock et al. 2019). The numbers of adults in these aggregations can vary substantially (Inkley 2012, Lee et al. 2014) and the aggregated individuals are often in contact with each other (Song and Lee 2021), especially when in large aggregations (JCB unpubl. data). During the overwintering period, studies have documented from 11.7 to 85.0% mortality (e.g., Bergh et al. 2017, Costi et al. 2017, Lowenstein and Walton 2018), although the potential impact of biotic agents on overwintering mortality has not been investigated.

The microsporidian pathogen, *Nosema maddoxi* Becnel, Solter, Hajek, Huang, Sanscrainte & Estep, is native to North America, Asia, and possibly Europe, where it infects pentatomids (Hajek et al. 2017, Kereselidze et al. 2020, AEH unpubl. data). *Nosema maddoxi*
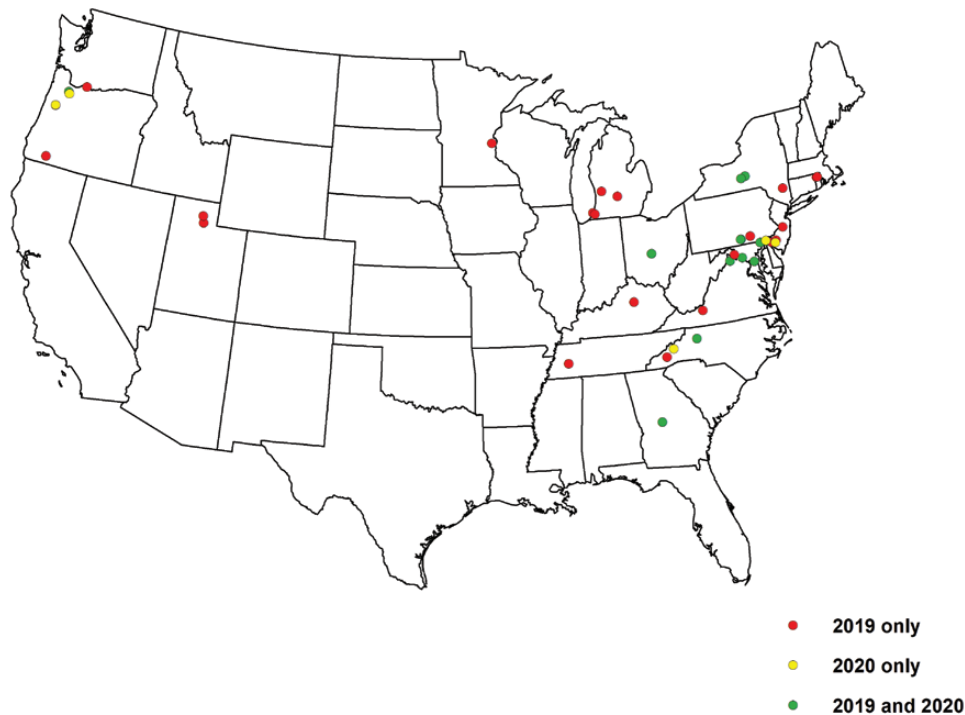
Fig. 1. Distribution of sites sampled in autumn 2019, spring 2020, or both, for comparisons of *Nosema maddoxi* infection levels.

is the best-known pathogen of *H. halys* and it also infects some native stink bug species in the United States (Hajek et al. 2017). In the United States, *H. halys* infected by *N. maddoxi* were first detected from declining laboratory colonies, and subsequently from field populations. In 2017 and 2018, *N. maddoxi* was documented from *H. halys* adults collected from 31 sites in 11 US states, averaging 18.9% infection (Preston et al. 2020a). *Nosema maddoxi* infects *H. halys* when ingested but generally does not cause acute disease (=fast mortality); yet, laboratory studies of infected *H. halys* revealed decreased longevity, egg production, and egg viability in adult females, and decreased nymphal survival (Preston et al. 2020b).

Given the importance of *H. halys* as an agricultural and nuisance pest in the United States, we were interested in the infection by and impact of *N. maddoxi* on its populations immediately before, during, and after the *H. halys* overwintering period. In particular, we investigated whether infection levels in *H. halys* populations changed from before overwintering to after overwintering (e.g., from autumn to the following spring). Preliminary studies (C. Preston, pers. comm.) indicated increased infection by *N. maddoxi* in *H. halys* adults at 2 of 3 sites in Pennsylvania in spring 2018 compared with autumn 2017. However, studies by Kereselidze et al. (2023) in the Guria region of the Republic of Georgia found that infection of *H. halys* adults by *N. maddoxi* was greater in autumn than in spring, although infection levels did not differ in the Samegrelo region. Greater infection in autumn compared with spring may be explained by the mortality of infected *H. halys* while overwintering. Mortality of *H. halys* overwintering outdoors (e.g., Haye et al. 2014, Costi et al. 2017, Lowenstein and Walton 2018) as well as in buildings (e.g., Chambers et al. 2019, Ciancio et al. 2021) occurs at levels that can be significant.

Here, we report the results of studies that investigated *N. maddoxi* prevalence in field populations of *H. halys* before overwintering in the autumn versus directly after overwintering the following spring, using specimens collected from 40 sites in 17 states. From 2020 to 2022, we also evaluated infection levels of fungal pathogens in *H.*

halys during the period between the onset and end of overwintering as well as post-overwintering, using adults that had settled in overwintering shelters in the autumn at 2 residences in Virginia. Although our initial focus was on *N. maddoxi*, we discovered another entomopathogen, *Colletotrichum fioriniae* Marcelino & Gouli, that was especially prevalent among *H. halys* that did not survive overwintering.

Materials and Methods

Sampling Adult *H. halys* in Autumn and Spring

Adult *H. halys* were collected from plants, buildings, and traps in autumn 2019 and/or spring 2020 at a total of 40 sites across 17 states (1–6 sites per state) (Fig. 1). At each site, collections were made from the same general areas in autumn (*n* = 35 sites) or spring (18 sites). Collections occurred from 5 September to 30 October, 2019 and from 15 April to 5 June, 2020 and the adult *H. halys* were held at –20°C before diagnosis. Sample sizes were between 15 and 30 adults per collection period per site. Across the 40 sites, 22 were sampled only in autumn 2019, 6 only in spring 2020 (overwintered adults), and 12 sites were sampled in both seasons.

Sampling Overwintering Adult *H. halys*
Field sites and overwintering shelters.

Adult *H. halys* that had voluntarily entered and settled in the wooden shelters described in Bergh et al. (2017) during their dispersal to overwintering sites were used for these studies. To deploy the shelters, wooden apple crates (38.1 × 29.8 × 45.7 cm) were attached to one another end to end using plastic cable ties to create a stack of 3 crates that was positioned upright (Fig. 2). One shelter was affixed to the inside bottom of the top crate in each stack via screws drilled through a flange that extended above the back of each shelter. A piece of brown corrugated plastic attached to the top of each stack overhung the opening, providing additional protection

Fig. 2. Deployment of an overwintering shelter for *Halyomorpha halys* within the top apple crate on a stack of wooden apple crates. Arrow indicates the shelter and its location.

from rain, and a cinder block in the bottom crate provided stability. Access to the interior of the shelters by *H. halys* that landed on them or walked to them was via a space between the top of the front panel and the overhanging roof, and through the bottom, which was open during deployment (see Bergh et al. 2017).

In 2020 and 2021, shelters were placed in the field during the week prior to 21 September, when the onset of mass movement of adult *H. halys* to buildings and overwintering sites in nature occurs (Bergh and Quinn 2018). Shelters were deployed against east-facing walls, on which the largest counts of invading adult *H. halys* had been recorded consistently in previous studies (Bergh and Quinn 2018). In 2020, shelters were deployed at each of 2 private residences that had experienced large annual invasions of adult *H. halys* in previous autumns. In 2020, 6 shelters were deployed at Site 1 (38°44′50.70″N, 78°06′54.14″W), which was a single-story home in a large clearing at the top of a forested knoll in Rappahannock Co., VA, and 5 were deployed at Site 2 (39°07′50.33″N, 78°13′29.57″W), a multistory residence in a clearing with woodlands on 3 sides, in Frederick Co., VA. The 2 sites were 43.7 km from each other. In 2021, shelters (*n* =

8) were deployed only at Site 1, due to the sale of the property at Site 2. In mid-November of both years, the bottom of each shelter was sealed with a fitted wood panel and the shelters were removed from the crates and transported to Virginia Tech's Alson H. Smith, Jr., Agricultural Research and Extension Center, near Winchester, VA, where they were stored in dark sealed plastic bags in a dark walk-in cooler at 4°C, to simulate winter.

Sampling protocol for overwintering adults.

To assess *H. halys* for pathogen infection, sub-samples of overwintering adults were removed from the same randomly-chosen shelter from each site in mid-December, mid-February, and mid-April during the winter of 2020–2021 and from site 1 in 2021–2022. These adults were brought to room temperature, with the intention of collecting living individuals. After 24 h, some individuals had begun to walk and these were collected. Those that did not exhibit this behavior within that period (which comprised most of the sub-sample), were evaluated by gently extending the legs laterally from the body with a dissecting needle. Adults that retracted their legs fully and immediately after this manipulation were considered alive and added to the sample of living individuals. After the mid-April collection of live adults from shelters in 2021, all remaining adults in the shelter from each site were moved into separate 30.5 cm³ screened cages (BioQuip, Rancho Dominguez, CA) in a greenhouse at ambient temperature and photoperiod. Each cage was provisioned with young, potted green bean plants (*Phaseolus vulgaris* L.), fresh green beans, dried seeds, nuts, young peaches, and water (see below). After 2 weeks, when living *H. halys* had become active and moved to plants or cage walls, all dead and moribund adults were collected from the bottom of each cage. Confirmation of the status of these adults was based on an assessment of whether they showed partial and lethargic movements when handled (=moribund) or no movement (=dead). The high mortality of the remaining caged adults observed subsequently prompted the collection of additional samples on 26 May and 3 June, 2021.

Following the collection of the 3 sequential sub-samples from individual shelters in winter–spring 2020–2021 and 2021–2022, subsequent sampling differed between the years. For brevity and clarity, Table 1 provides details of the sampling timeline for each year, including all sample sizes, and footnotes explaining the reasons underlying the differences. All individuals (live, and/or dead, and moribund) collected at each sampling time point were placed in labeled bags and held in a freezer at –80°C. These were shipped with cold packs via overnight courier to AEH, where they remained frozen until evaluation for pathogen infection.

Pathogen Diagnosis

Halyomorpha halys individuals that were collected, frozen, and diagnosed had no external fungal growth. Each individual *H. halys* was diagnosed for *N. maddoxi* as described in Preston et al. (2020b). Briefly, *H. halys* adults were placed in 0.05% Tween and macerated with a micropestle. Samples were observed at 400× under phase contrast to detect the presence of *N. maddoxi* or *C. fioriniae* spores. After *C. fioriniae* was first found, it was isolated on potato dextrose agar and deposited in the USDA ARSEF culture collection (isolates # 14595 & 14596). A separate paper on the identification of *C. fioriniae* from *H. halys* is being prepared, in which positive results from Koch's postulates will also be presented (J.B. González unpubl. data). *Colletotrichum fioriniae* spores differ markedly in width and length from those of *N. maddoxi*. *Nosema maddoxi* spores were very consistent in shape and size: oblong with a length × width of

Table 1. Timeline of collections of adult *Halyomorpha halys* for assessments of pathogen infection, 2020–2021 and 2021–2022

Year	Site	Collection date	Shelter or cage[a]	Number *H. halys* adults collected		
				Live	Moribund	Dead
2020–2021	1	8 Dec	Shelter[b]	50		
	2	8 Dec	Shelter[b]	30		
	1	9 Feb	Shelter[b]	52		
	2	9 Feb	Shelter[b]	30		
	1	14 Apr	Shelter[b]	84		
	2	14 Apr	Shelter[b]	35		
	1	28 Apr	Cage[c]		38	268
	2	28 Apr	Cage[c]		14	68
	1	26 May	Cage[c]	30		114
	2	26 May	Cage[c]	30		36
	1	3 Jun	Cage[c]	36		176
	2	3 Jun	Cage[c]	65		68
2021–2022	1	15 Dec	Shelter[d]	53		
	1	16 Feb	Shelter[d]	30		
	1	11 Apr	Shelter[d]	62		
	1	8 Apr	Cage[e]		44 (DM)	
	1	8 Apr	Cage[e]		67 (DM)	
	1	6 Jun	Cage[f]		68	637
	1	7 Jun	Cage[g]		7	148
	1	8 Jun	Cage[h]		3	15
	1	8 Jun	Cage[i]		43	846

[a]Wooden overwintering shelter used to collect adult *H. halys* in autumn and screened 30 cm³ cage used to hold *H. halys* adults under ambient conditions in a greenhouse after removal from shelters.

[b]One shelter from Sites 1 and 2, from which sub-samples of live adults were collected within 24 h after removal from cold storage on 8 Dec, 9 Feb, and 14 Apr 2020–2021.

[c]Cage containing all adults remaining in the shelter from each site after the 2021 Apr 14 living sub-sample was taken. Collections on 28 Apr represent moribund and dead adults from each shelter that had accumulated from when shelters were colonized, through time at 4°C and until 14 d after cages had been moved to the greenhouse; 14 days was the time required by living *H. halys* to become active. Collections on 26 May represent sub-samples of living and dead adults from each cage in the greenhouse. The sub-sample on 26 May was prompted by observations of high mortality in cages. The sample on 3 Jun represents all adults (living and dead) remaining in each cage.

[d]One shelter from Site 1, from which sub-samples of live adults were collected within 24 h after removal from cold storage on 15 Dec, 16 Feb, and 11 Apr 2021–2022. After collection of live adults on 2022 Apr 11, adults remaining in that shelter were lost. Consequently, adults taken from 2 other shelters deployed at Site 1 were used for comparisons of infections (see footnote e).

[e]Two shelters were removed from cold storage on 8 Apr and all adults from each were placed in separate cages for 1–2 wks, after which moribund and dead adults were collected (but were merged for each cage = DM).

[f-i]One or two shelters were removed from cold storage on each date shown and all adults were placed in separate cages for 1–2 wks, after which all dead adults were collected. Different footnotes indicate different shelters.

4.72 ± 0.05 × 2.19 ± 0.03 µm (Hajek et al. 2017). *Colletotrichum fioriniae* spores from our samples were oblong but variable in size (length × width: 9.34 ± 0.29 × 3.91 ± 0.10 µm; range 6–15 × 3–5) and always larger than spores of *N. maddoxi* (AEH unpubl. data). For the study comparing infections between individuals collected in the autumn and/or the spring, 1,351 *H. halys* were diagnosed, while for the study that compared infections during and after the overwintering period, 1,220 and 501 *H. halys* were diagnosed in 2020–2021 and 2021–2022, respectively. In 2020–2021, all samples collected were diagnosed while in 2021–2022, all samples from Dec. through April were diagnosed while at least 100 individuals were diagnosed from each cage for June 2022 samples.

Data Analysis

The percentages of *H. halys* adults infected with *N. maddoxi* in autumn versus spring were compared using Proc Glimmix (SAS 2021). To compare infection levels between live and dead adults when overwintering, comparisons utilized the percent *N. maddoxi* infections in April. For 2022, dead and moribund adults from the same shelter from which live adults were collected in April were lost,

so dead and moribund adults from 2 other shelters maintained at the same location and collected in April were jointly substituted. Analyses comparing pathogens associated with living adults (while overwintering) versus dead adults (that had accumulated throughout overwintering) were conducted using Proc Glimmix. Infection levels for dead and moribund were merged as there was no significant difference between them (*N. maddoxi*: $F_{1,4} = 4.25$, $P = 0.1082$; *C. fioriniae*: $F_{1,4} = 1.26$, $P = 0.3247$).

Results

Infection in *H. halys* during Autumn versus Spring

Adult *H. halys* infected by *N. maddoxi* were found at 18 of the 35 sites sampled across 17 states in autumn 2019 (Fig. 3). Among all sites, percent infection in autumn averaged 6.8 ± 1.7% and among sites with positive detections, the percent infection averaged 13.3 ± 2.4% (range = 3.3–40.0%). In spring 2020, *N. maddoxi* was detected in *H. halys* from 11 of the 18 sites sampled across 10 states, with infection averaging 7.6 ± 2.5% among all sites and 13.4 + 2.5% at the positive sites (range = 3.3–36.0%). Although

low infection levels were recorded at some sites from both autumn and spring sampling, *N. maddoxi* was confirmed in *H. halys* for the first time in Delaware, Georgia, Michigan, New Jersey, Rhode Island, and Tennessee. At the 12 sites across 8 states from which samples were collected in both autumn 2019 and spring 2020, the average *N. maddoxi* infection in autumn (12.3 ± 3.9%) did not differ significantly from that in the spring (8.2 ± 3.4%) ($F_{1,11}$ = 3.03; P = 0.1098). Across all collections, *N. maddoxi* infection levels were consistently and significantly higher in the Mid-Atlantic region (n = 27 sites, 10.2 ± 2.2%) than in the other regions sampled (n = 26 sites, 3.6 ± 1.4%) (chi-squared = 25.9319; P < 0.00001).

Infection among Overwintering and Post-overwintered *H. halys*

In 2020–2021 and 2021–2022, *N. maddoxi* infection levels in living adult *H. halys* did not differ significantly among the samples from mid-December, mid-February, and mid-April ($F_{2,4}$ = 1.15; P = 0.4036). *Nosema maddoxi* infection levels among living adults during this period averaged 13.4 ± 4.5%. In early-mid April, when overwintering was ended, *N. maddoxi* infection levels were significantly higher in dead (33.4 ± 10.8%) than live (11.1 ± ± 5.6%) adults for pooled years and sites ($F_{1,2}$ = 33.70; P = 0.0284) (Supplementary Fig. 1).

Surprisingly, a second pathogen, *C. fioriniae*, was prevalent in moribund and dead individuals within shelters. *Colletotrichum fioriniae* was never found infecting live *H. halys* collected from shelters in December, February, or April in 2020–2021 or 2021–2022 (Supplementary Fig. 1). Infection levels by *C. fioriniae* in the dead and moribund *H. halys* collected in April from overwintering shelters in 2021 and 2022 averaged 46.7 ± 7.8% (range: 36.0–68.3%). Among moribund and dead *H. halys* that had accumulated in shelters, *C. fioriniae* co-occurred with *N. maddoxi* in 11.1 + 5.9% of infected moribund and dead adults (Supplementary Fig. 1).

In 2021, when infection in caged post-overwintering adults was evaluated, *N. maddoxi* infections increased with time in cages (y = 0.0792e$^{0.0281x}$; r^2 = 0.4561) (Fig. 4). *Colletotrichum fioriniae* infections were 0–4.6% in living *H. halys* in cages in May and June 2021. Percent *C. fioriniae* infections among the dead adults in die-offs occurring in May and June ranged from 9.7% to 27.8% but no significant trend across time occurred, except that these infection levels were much lower than among dead *H. halys* from overwintering shelters.

The percentage of dead and moribund adults in the 3 shelters removed from cold storage in April 2022 (after 5 months of cold storage), averaged 34.6 ± 4.8%. Among the 4 additional shelters that remained at 4°C until early June (approximately 7 months of cold storage), mortality was significantly greater by early June (84.5 ± 2.0%; $F_{1,4}$ =71.84; P = 0.0011). However, although mortality in shelters at 4°C more than doubled between approximately 5 and 7 months of cold storage, infection levels of *N. maddoxi* and *C. fioriniae* in dead adults decreased significantly, from 76.3 ± 3.2% in April, to 43.7 ± 7.4% in June, and the percent of *H. halys* dying that were not infected with either pathogen increased from 23.7 ± 3.2% for April samples to 56.3 ± 7.4% for June samples ($F_{1,4}$ = 44.29; P = 0.0026).

Discussion

Our population-level results did not demonstrate a consistent significant trend in changes in *N. maddoxi* infection levels from before (autumn) to after (spring) overwintering. These results agree with those from the Republic of Georgia, where *N. maddoxi* infection levels did not change significantly from autumn to spring in one region, although *N. maddoxi* infection levels were higher in autumn than spring in a second region where the density of *H. halys* was higher (Kereselidze et al. 2023). This trend could be explained by the death of infected *H. halys* while overwintering, which would result in fewer infected *H. halys* surviving. Our overwintering studies demonstrated
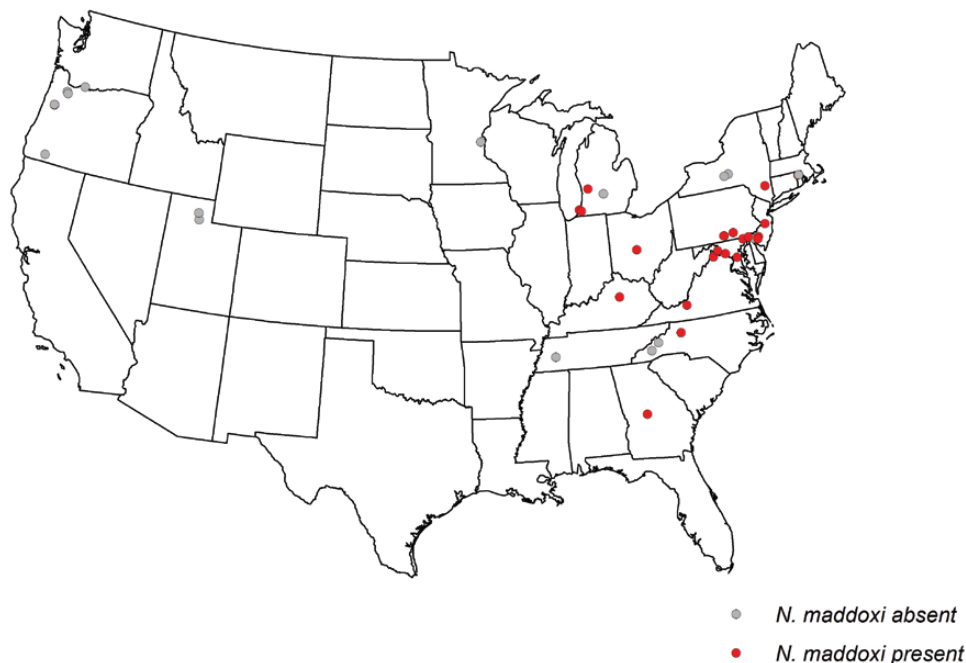

N. maddoxi absent

N. maddoxi present

Fig. 3. Presence/absence of *Nosema maddoxi* in *Halyomorpha halys* collected from sites sampled in autumn 2019 and/or spring 2020.
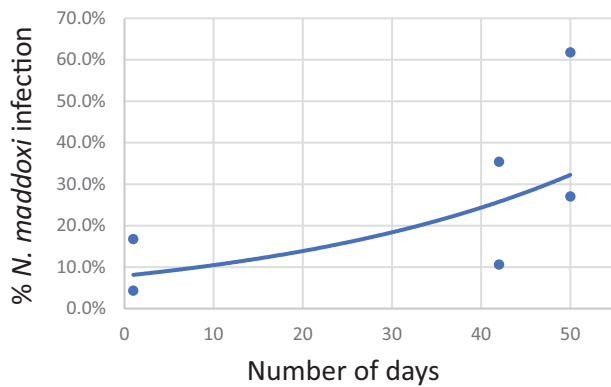
Fig. 4. Increase in *Nosema maddoxi* infection levels starting with percent infection among living overwintering adults in shelters in April 2021. Adult *Halyomorpha halys* were moved to greenhouse cages (day 1) and percent *N. maddoxi* infection post-overwintering (living and dead) increased through 2 die-offs observed 26 May (after 42 d in cages) and 3 June (50 d in cages).

that more *H. halys* that died while overwintering contained spores of *N. maddoxi* and/or *C. fioriniae* than *H. halys* surviving overwintering, strongly suggesting that adults infected with these fungal pathogens had a greater chance of mortality during overwintering. Among individuals of the coccinellid *Harmonia axyridis* (Pallas) infected with the ectoparasitic fungus, *Hesperomyces virescens* Thaxter, higher mortality in infected versus healthy insects occurred while overwintering (Knapp et al. 2022). These experimental studies with ladybeetles suggested that a mix of energy exhaustion and decreased immune response were responsible for the increased mortality of infected *H. axyridis* while overwintering.

Infection levels of *N. maddoxi* in *H. halys* 2021 increased over time after overwintering, causing epizootics. In this case, post-overwintering *H. halys* were provided with food on which they could regain energy. However, these adults were confined in cages in a greenhouse for >1 month and, in such conditions, it is well known that *N. maddoxi* transmission among individuals occurs (Hajek et al. 2017). In the absence of other stressors, infection by this chronic pathogen does not always cause *H. halys* mortality (Preston et al. 2020b). However, it is possible that the increased age of adults that had survived overwintering since the previous autumn may affect their immune response to pathogen infection, thus potentially explaining increased infection and death post-overwintering. Indeed, the overwintering generation of adult *H. halys* is known to be more susceptible to insecticide exposure than the subsequent F1 and F2 adult generations (Leskey et al. 2014), indicating their depleted state after overwintering.

Our study of infection by pathogens during and after overwintering was conducted with *H. halys* maintained at constant 4°C for 5 months to mimic overwintering conditions, but this did not provide the variable temperature and moisture levels that would occur when overwintering outdoors and perhaps to a lesser extent the conditions experienced by those overwintering in protected spaces in buildings. In addition, in our study all *H. halys* ended overwintering at the same time, while in nature adult *H. halys* leaving overwintering aggregations occurs over a protracted period (Skillman et al. 2018). Studies of overwintering *H. halys* found that adults held for 20 weeks at 4°C showed reductions in energy reserves and survival (Taylor et al. 2017, Skillman et al. 2018), as would have occurred in this study. We found that while *H. halys* were kept at constant 4°C for 5 months, levels of *N. maddoxi* infection in live adults did not change within the overwintering period. The

dead and moribund adults that were collected from shelters when overwintering ended are assumed to have accumulated throughout the period in cold storage, and possibly also during the 2-month period before the shelters were moved from the field to cold storage. Levels of *N. maddoxi* in dead and moribund adults were greater than in live adults during the overwintering period, and we hypothesize that *N. maddoxi* infections negatively impacted survival of some of the *H. halys* while overwintering.

Levels of mortality of *H. halys* held at 4°C until June in 2022 were very high compared with mortality of individuals in cold storage until April. Levels of mortality of uninfected *H. halys* increased among adults held in shelters at 4°C until June, compared with April. We hypothesize that increased mortality was due to the inability to survive the cold for such a prolonged period, as would be predicted based on Taylor et al. (2017).

Molecular methods identified *C. fioriniae* as the entomopathogen that had infected and killed many of the dead *H. halys* from overwintering shelters and, albeit in lower numbers, post-overwintering bugs in 2021 that died the following May and June. This pathogen had not previously been reported from *H. halys*, and Koch's postulates were used to demonstrate its pathogenicity (AEH unpubl. data). *Colletotrichum fioriniae* was never found infecting live *H. halys* during overwintering sampling and was rarely found in live *H. halys* post-overwintering. High *C. fioriniae* infection levels mostly occurred among *H. halys* that had died within shelters during the period between settling in shelters through overwintering. These results may suggest that *H. halys* infected with *C. fioriniae* did not survive very long (as opposed to *N. maddoxi* which generally causes more of a chronic disease) as would be consistent with our pathogenicity tests. Low percentages of *C. fioriniae* infections in well-fed living *H. halys* post-overwintering could suggest that hosts not stressed by low temperatures and lack of food might better prevent or survive *C. fioriniae* infections.

Colletotrichum fioriniae (previously known as *C. acutatum* J.H. Simmonds or *C. acutatum* var. *fioriniae* J.A.P. Marcelino & S. Gouli) is a well-known fungal plant pathogen (some diseases that are caused are called anthracnose) and plant endophyte, occurring worldwide (Damm et al. 2012, Martin and Peter 2021). However, it is also known to cause epizootics in another North America hemipteran, the elongate hemlock scale (*Fiorinia externa* Ferris) (Marcelino et al. 2008). Laboratory bioassays with *C. fioriniae* also demonstrated infection in larvae of the lepidopteran, *Spodoptera exigua* (Hübner), the whitefly *Bemisia argentifolii* Bellows & Perring, and the thrips *Frankliniella occidentalis* Pergande (Marcelino et al. 2009). In a study of whether *H. halys* can act as a vector of the fungal disease anthracnose to tomato plants, Voshell (2015) recovered what was reported as *C. acutatum* spores from stylets and legs of *H. halys* feeding on infected tomatoes but reported low mortality of *H. halys* exposed to infected tomatoes. The fungal isolate investigated by Voshell (2015) was likely *C. fioriniae*, based on the available partial GAPDH nucleotide sequence (although it was reported as *C. acutatum*). Thus, *H. halys* adults entering overwintering aggregations in autumn could be contaminated with *Colletotrichum* spores on their bodies, acquired by feeding on infected plants, and the pathogen could have subsequently infected and killed the aggregated overwintering adults. Our finding *C. fioriniae* infections in adults from both sites in 2020–2021 and in the one site sampled the following season, suggests that infections by this pathogen among overwintering *H. halys* are not uncommon in this region.

While our surveys in the autumn and spring of 2019–2020 yielded the first confirmed detections of *N. maddoxi* in 6 states, indications of its presence in Delaware and New Jersey had been

suggested earlier. *Nosema maddoxi* was first noted in 2012 in an *H. halys* laboratory colony in Florida that was initiated from bugs from Delaware (Hajek et al. 2017). At US field sites, *N. maddoxi* was probably first discovered (as an unnamed microsporidian species) in New Jersey in 2014 by Dr. Bryan Petty working with Dr. Anne Nielsen at Rutgers (A.E.H. unpubl. data). In Oregon and Utah, *N. maddoxi* was not found during this study but had been detected previously (Preston et al. 2020a), possibly indicating a patchy distribution in those states and/or low infection levels, making its detection less likely.

In summary, we found no differences in *N. maddoxi* infection levels among *H. halys* adults collected in the field in autumn versus spring and we found that 2 fungal pathogens infected and killed *H. halys* while overwintering and post-overwintering. The microsporidian, *N. maddoxi*, was always more prevalent in dead *H. halys* in overwintering shelters compared with bugs that survived overwintering. The fungus, *C. fioriniae*, is reported for the first time as a pathogen of *H. halys*. High levels of infection by *C. fioriniae* were found among bugs that did not survive overwintering. After overwintering, surviving bugs that were caged with food in a greenhouse showed steadily increasing levels of *N. maddoxi* infection over 50 d, but *C. fioriniae* infection levels were lower than in the adults that had died in overwintering shelters. We conclude that these 2 pathogens infecting *H. halys* contribute to mortality during and after overwintering, and this underlies some of the reported overwintering mortality. Activity of pathogens during and after overwintering caused reductions in the numbers of adult *H. halys* surviving to reproduce in spring, and *N. maddoxi* infections would also decrease the fecundity of survivors (Preston et al. 2020b).

Author Contributions

Ann Hajek (Conceptualization-Equal, Data curation-Equal, Formal analysis-Lead, Funding acquisition-Lead, Project administration-Lead, Resources-Equal, Supervision-Equal, Writing – original draft-Lead, Writing – review & editing-Supporting), Samuel Brandt (Methodology-Lead, Writing – review & editing-Supporting), Jennifer Gonzalez (Methodology-Equal, Validation-Equal, Writing – review & editing-Supporting), J. Christopher Bergh (Investigation-Equal, Project administration-Equal, Supervision-Equal, Writing – original draft-Supporting, Writing – review & editing-Equal)

Supplementary Material

Supplementary material is available at *Journal of Insect Science* online.

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