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Abstract

Halyomorpha halys (Stål), the brown marmorated stink bug, is an invasive and highly polyphagous insect that has caused serious economic injury to specialty and row crops in the United States and Europe. Here, we evaluated the effects of marking adult and nymphal *H. halys* with four different colors of fluorescent powder (Blaze Orange, Corona Pink, Horizon Blue, and Signal Green) on mobility and survivorship in laboratory bioassays. Adults and nymphs were marked using liquified fluorescent powder solutions and allowed to dry prior to bioassay. The presence of the marking solution had no significant effects on adult or nymphal mobility, adult survivorship, nymphal development, or adult flight capacity. We also evaluated the persistence of neon marker applied to the pronotum of *H. halys* adults and found this technique remained detectable for 2 wk under field conditions. Although both marking techniques are inexpensive, persist for ≥1 wk, and do not affect mortality, the neon marker method is more time-consuming, taking ~12 times longer to mark 50 adult *H. halys* compared with the liquified fluorescent powders. Thus, we would recommend using fluorescent powders for large-scale mark-release-recapture studies.

Keywords

fluorescent powder, mark-release-recapture, brown marmorated stink bug, dispersal

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is a serious invasive agricultural pest native to Asia (Lee et al. 2013a). With a host range of more than 170 host plant species including orchard crops, small fruit, vegetables, field crops, and ornamental plants, *H. halys* has inflicted serious economic injury in the United States and Europe (Leskey and Nielsen 2018). In addition, adult *H. halys* is a nuisance pest for homeowners, as large numbers disperse to (Hancock et al. 2019) and overwinter within residential buildings (Inkley 2012). While monitoring and biosurveillance tools are available for detecting both nymphs and adults (Short et al. 2017; Acebes-Doria et al. 2017, 2020), they do not provide information on movements of individuals, merely the endpoint where they were captured. Moreover, *H. halys* disperse between and among host plant species throughout the growing season (Leskey and Nielsen 2018), but the patterns of dispersal in agroecosystems are poorly understood.

Mark-release-recapture experiments can be used to examine animal dispersal behavior and movement patterns within landscapes. Entomologists have used fluorescent powders for close to a century to investigate insect dispersal (Darling 1925, Hagler and Jackson 2001). Fluorescent powders are inexpensive, available in a wide variety of colors, and can be rapidly applied to large numbers of insects (Stern and Mueller 1968, Crumpacker

1974, Beier et al. 1982). Several laboratory and field studies have shown that fluorescent marking has no detrimental effects on survivorship or behavior (Crumpacker and Williams 1973, Corbett and Rosenheim 1996, Hagler and Jackson 2001, Adams et al. 2017, Rice et al. 2017, Kirkpatrick et al. 2018). However, other studies found negative effects of fluorescent powders on insect sensory, mobility, and survivorship (Meyerdirk et al. 1979, Cook and Hain 1992, Pardo et al. 1996, Reid and Reid 2008, Stephens et al. 2008, Dickens and Brant 2014). Therefore, prior to field evaluations, the effects of specific markers should be evaluated to ensure marking does not have a negative effect on the survivorship or behavior of the target species (Southwood 1978, Hagler and Jackson 2001).

Mass-marking techniques using fluorescent powders have been useful for understanding dispersal and other movement behaviors for many taxa, including Diptera (Weldon 2005, Rice et al. 2017, Kirkpatrick et al. 2018), Coleoptera (Naranjo 1990), Lepidoptera (Vilarinho et al. 2006, Adams et al. 2017), Hymenoptera (Garcia-Salaza and Landis 1997), and Hemiptera (Bancroft 2005, Tillman et al. 2009, Kelly et al. 2014, Walsh et al. 2016) and none of these studies revealed negative impacts on insect behavior and survivorship. To better understand their behavior, *H. halys* have been marked with harmonic radar tags (Lee et al. 2013b, 2014; Blaauw et al. 2017; Kirkpatrick et al. 2019b), proteins (Blaauw et al. 2016, 2017), and fluorescent powders, but this latter effort only tested detectability of *H. halys* using freshly killed marked individuals and either handheld lasers or unmanned aerial vehicles (UAVs) to recover individuals (Rice et al. 2015, Stumph et al. 2019).

We therefore evaluated the effect of marking adult and nymphal *H. halys* with four commonly used fluorescent powders and two colors of neon pens. Our specific objectives were to quantify: 1) effects of marking on survival of adults and nymphs, vertical and horizontal mobility of adults and nymphs, and flight capacity of fluorescent-marked adults, and 2) time required for marking methods and persistence of neon pen marks on adults released in the field. Ultimately, these studies will provide confidence in selection of marking methods for future mark-release-recapture studies of *H. halys*.

Materials and Methods

Fluorescent Marking Studies

Halyomorpha halys colony

To establish a laboratory colony of *H. halys*, we collected adults from wild *Paulownia tomentosa* (Thunb.) Steud. and *Ailanthus altissima* (Mill.) Swingle in Kearneysville, WV from September to October 2018. We placed these adults in screen cages (30 cm^3; DP1000, BugDorm, BioQuip, Rancho Dominguez, CA) with food (carrot, peanuts, sunflower seeds, and sundried tomato) and water (moistened cotton dental wicks) in a climate-controlled room at 25°C, 60% RH, and 16:8 (L:D) h. Adults mated and laid eggs in cages and we reared nymphs under the same conditions to fourth or fifth instars in screen cages (30 cm^3; DP1000, BugDorm, BioQuip) with similar food, water, and climatic conditions.

Application of Fluorescent Powders

Fluorescent powders (DayGLO Color Corporation, Cleveland, OH) used for all assays included Horizon Blue (#2141), Signal Green (#1948), Blaze Orange (#1874), and Corona Pink (#2007). We mixed powders with deionized water (0.5 g fluorescent powder/100 ml deionized water) in spray bottles to create a liquified fluorescent powder solution. We placed groups of *H. halys* (~50 individuals) in deli-cups (946 ml; Fabri-Kal Corporation, Kalamazoo, MI) and sprayed ~8.8 ml of fluorescent solution and gently swirled the solution 8–10 times to coat *H. halys* and subsequently drained excess liquid. We removed marked insects from cups and placed onto paper towels in screen cages to dry for ~5 min (Fig. 1A and B).

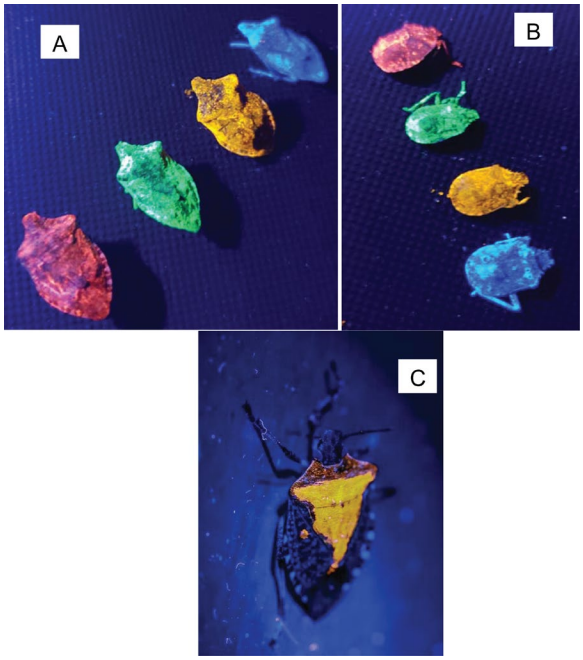

Fig. 1. Adult (A) and nymph (B) *Halyomorpha halys* marked with various colors of fluorescent powder under UV light, and (C) pronotum of adult *H. halys* marked with a yellow neon pen.

Impact of Fluorescent Marking on *H. halys* Adult Survivorship and Nymph Development

We monitored marked and unmarked adults (sex ratio = 1:1) and fourth and fifth instars each day for 10 d and recorded their status (alive, moribund [immobilized, typically lying on dorsum and with uncoordinated leg movements] or dead). For nymphs, we used newly emerged marked and unmarked control nymphs and monitored the number of days until molting occurred. For the duration of the assays, marked adults and nymphs were held in individual clear deli-cup containers in a climate-controlled room (conditions, containers, food, and water as above).

For nymphs, we detected no significant difference in the development time between fourth and fifth instars; thus, we pooled data for analysis for both instars. We conducted 10 replicates (~50 individuals per replicate) for both adults and nymphs. Adult survivorship data did not meet assumptions of normality; therefore, we used a nonparametric Kruskal–Wallis test. In contrast, we analyzed nymphal development using analysis of variance (ANOVA) because data met assumptions of normality. We used JMP (v.13; SAS Institute, Cary, NC, 2016) to analyze this and subsequent experiments.

Horizontal Movement of Fluorescent Powder-Marked *H. halys* Adults and Nymphs

We measured horizontal walking capacity of marked fourth and fifth instars, and adults (Lee et al. 2013b) movements in real time using Noldus Ethovision software (version 3.1, Noldus Information Technology Inc., Leesburg, VA). Using five arenas, one marked adult or nymph of each color (as described above) and one unmarked control were tracked simultaneously in Petri dish arenas (10 cm diameter; 3 cm tall) using a camera (Re-350, Canon, Inc., Tokyo, Japan) suspended above the arenas for a duration of 1 h. To aid in detection and tracking by software, we conducted tests in a darkened climate-controlled room (25°C and 60% RH) with the arenas backlit using fluorescent lights. Because horizontal mobility of fourth and fifth instars did not differ significantly, we combined data for both instars. For both adults and nymphs, we conducted 10 replicates with each set of five arenas considered a replicate. To meet assumptions of normality, we log-transformed data for distance moved, angular velocity, and speed data and analyzed them using one-way ANOVA for both adults and nymphs.

Vertical Mobility of Fluorescent Powder-Marked *H. halys* Adults and Nymphs

We evaluated vertical mobility of marked fourth and fifth instar nymphs and adults compared with unmarked controls (Lee et al. 2013b). We placed marked or unmarked individuals inside and at the base of clear polycarbonate cylinders (30 cm tall × 7 cm diameter) in a climate-controlled room (25°C and 60% RH) and recorded distance climbed over 15 min using marked measurements on the cylinders. Once an individual reached the top of the cylinder, we inverted the cylinder to enable continuous movement of the adult or nymph for the duration of the experiment. We conducted 10 replicates for both adults and nymphs (no difference between mobility of fourth and fifth instars, data were combined) with one set of marked individuals of each color and unmarked control each tested in separate cylinders constituting a replicate. To meet assumptions of normality for nymphs, we square root transformed the data and analyzed it with one-way ANOVA.

Flight Capacity of Fluorescent Powder-Marked *H. halys* Adults

We measured flight capacity of marked and unmarked adults using six flight mills following methods presented by Lee and Leskey (2015) in the laboratory (25°C, 60% RH, 16:8 [L:D] h and 1,150 lux). We used DASYLab (Measuring Computing, Norton, MA) to record the number of rotations per flight, total distance, and flight duration. Adults were tethered to flight mills using a droplet of glue from a low-temperature glue gun applied to the head of an insect pin and gently pressed onto the center of the pronotum of each individual. We then inserted the point of the insect pin into the tip of one end of rotation arm of the flight mill to position the insect for flight and placed a counterweight on the opposite end of the rotation arm. Trials began at 0830 h after gently blowing each tethered *H. halys* to encourage flight. Thereafter, adults remained undisturbed in the laboratory room for 22 h. We conducted 20 replicates, each consisting of a marked individual for each color and an unmarked control. To meet assumptions of normality, we log-transformed flight distances and analyzed the data with one-way ANOVA.

Neon Pen Marking Method for *H. halys* and Field Recovery

In field trials, we marked the pronota of *H. halys* adults with neon pens and measured its persistence in Lancaster, PA in September 2013. We hand-collected *H. halys* adults from soybean fields, anaesthetized them with CO_2, then applied marks to the entire pronotum with either yellow (Fig. 1C) or pink neon markers (Sharpie, Oak Brook, IL). Marked adults were placed in mesh cages (35 × 35 × 61 cm, Bioquip, Rancho Dominquez, CA) in the field after sunset (~2300 h). We then removed the top of the cage, positioned foliage from a nonhost plant placed inside, and allowed adults to exit the cage. We released a total of 1,250 adult *H. halys* bearing pink and 1,250 adults bearing yellow neon marks 2 m into a soybean field and 2 m into a mixed coniferous wooded area, respectively. Using the methods of Rice et al. (2015), we scouted the soybean field and wooded area using handheld UV spotlights (Labino, Solona, Sweden) at 48 h, 1 wk, and 2 wk after release to examine persistence of the neon markings on *H. halys* adults.

Results and Discussion

Our results demonstrate that marking adult and nymph *H. halys* with fluorescent powder solution had no detectable effect on adult survivorship or nymphal development. Survivorship of fluorescent-marked adults was not different over the 10-d period ($\chi^2 = 5.933$; df = 4; $P = 0.204$) among all colors and unmarked *H. halys* (Fig. 2A). For nymphs, development time was not statistically different ($F = 1.763$; df = 4, 45; $P = 0.153$) among marked and unmarked individuals (Fig. 2B).

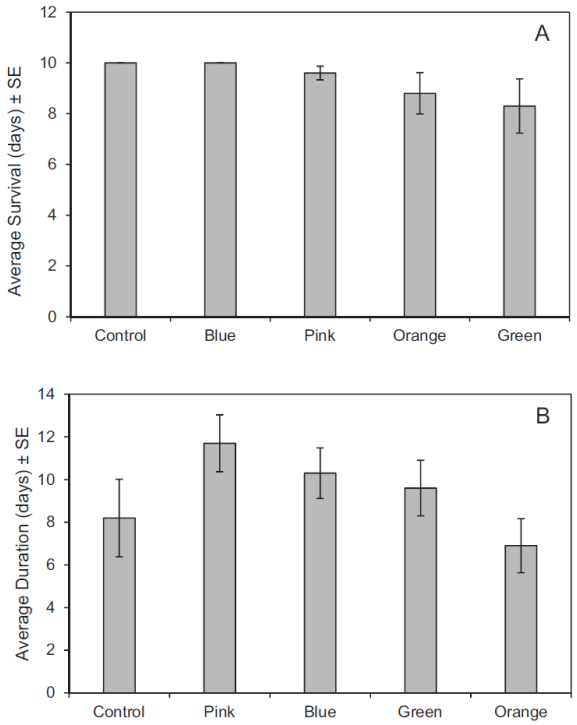

Fig. 2. Outcomes of laboratory experiments investigating the influence of four different colors of fluorescent powder and an unmarked control on *Halyomorpha halys* (A) adult survivorship and (B) instar duration (no statistical differences among treatments; A: Kruskal–Wallis, $P = 0.204$, B: ANOVA, $P = 0.153$).

For adults, horizontal walking distance ($F = 1.210$; df = 4, 45; $P = 0.320$), angular velocity ($F = 1.079$; df = 4, 45; $P = 0.379$), and walking speed ($F = 2.579$; df = 4, 45; $P = 0.630$) were not significantly different for marked and unmarked individuals. Similarly, horizontal walking distance ($F = 0.672$; df = 4, 45; $P = 0.615$), angular velocity ($F = 0.202$; df = 4, 36; $P = 0.936$), and walking speed ($F = 1.079$; df = 4, 45; $P = 0.379$) were not different for marked and unmarked nymphs (Table 1). Fluorescent-marked and unmarked *H. halys* adults ($F = 1.386$; df = 4, 45; $P = 0.254$) and nymphs ($F = 1.217$; df = 4, 45; $P = 0.317$) did not differ in vertical distances climbed (Fig. 3A and B). However, it is likely that adults and nymphs walk different distances under field conditions.

Table 1. Average horizontal walking distance (cm ± SEM), angular velocity (degrees/s ± SEM), and speed (cm/s ± SEM) of fluorescent-marked or unmarked adult and nymph *Halyomorpha halys*

	Average distance moved (cm)*a*		**Average angular velocity (degrees/s)***b*		**Average speed (cm/s)***c*	
Treatment	**Adults**	**Nymphs**	**Adults**	**Nymphs**	**Adults**	**Nymphs**
Control	558.5 ± 292.9	2,252.9 ± 598.3	33.6 ± 7.3	23.4 ± 12.8	0.2 ± 0.1	0.6 ± 0.2
Blue	912.4 ± 369.8	2,296.0 ± 617.9	20.9 ± 5.1	20.5 ± 11.3	0.3 ± 0.1	0.6 ± 0.2
Pink	1,128.5 ± 384.0	1,417.8 ± 347.9	16.2 ± 7.2	16.2 ± 7.4	0.3 ± 0.1	0.4 ± 0.1
Green	728.3 ± 207.5	1,966.9 ± 609.7	10.9 ± 2.3	75.1 ± 58.5	0.2 ± 0.1	0.5 ± 0.2
Orange	623.9 ± 107.1	2,747.8 ± 720.4	22.1 ± 8.2	50.8 ± 25.4	0.2 ± 0.03	0.8 ± 0.2

There were no statistical differences among treatments for adults or nymphs in any tests.

[a]Adults: *P* = 0.320 and nymphs: *P* = 0.615.
[b]Adults: *P* = 0.379 and nymphs: *P* = 0.936.
[c]Adults: *P* = 0.630 and nymphs: *P* = 0.379.

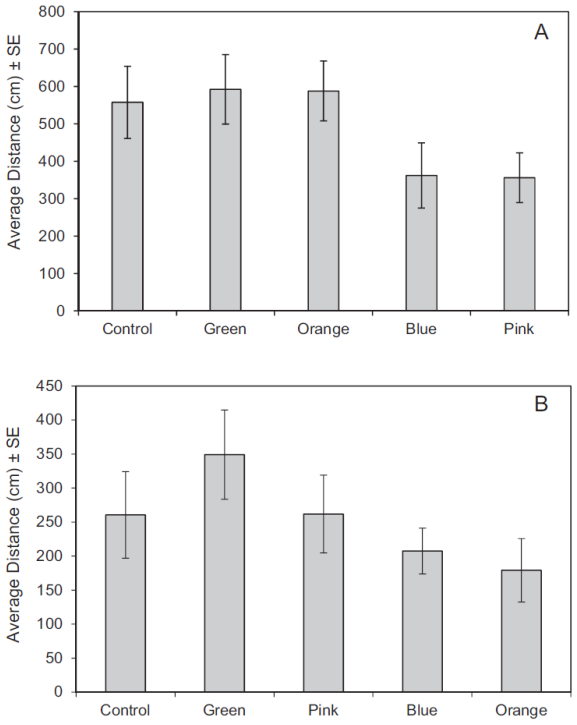

Fig. 3. Outcomes of laboratory experiments investigating the vertical distance (cm ± SE) climbed of (A) adult and (B) nymph *Halyomorpha halys* marked with four different colors of fluorescent powder and an unmarked control (no statistical differences among treatments; A: ANOVA, *P* = 0.245 and B: ANOVA, *P* = 0.317).

Additionally, we observed no difference in flight distance for marked and unmarked adults (Fig. 4; *F* = 2.0; df = 4, 98; *P* = 0.938). These results align well with previous results (Lee and Leskey 2015), which showed that unmarked males and females flew on average 2,442 m/22 h and 2,083 m/22 h, respectively. Previously, flight behavior of adult *H. halys* on flight mills and in the field was not found to be influenced by harmonic radar tags (Lee et al. 2013b). Thus, we anticipate that fluorescent marking on adults should similarly have little or no impact on their flight capacity under field conditions and could be used in conjunction with harmonic radar tags.

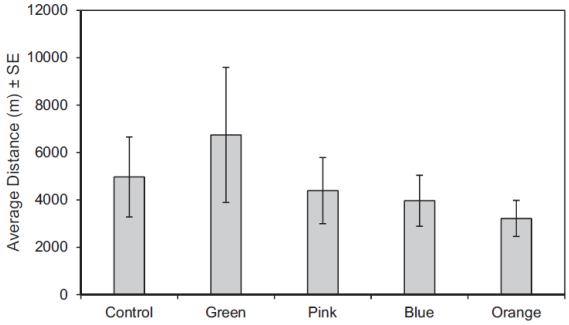

Fig. 4. Average distance (m ± SE) flown by adult *Halyomorpha halys* marked with four different colors of fluorescent powder and an unmarked control. There was no significant difference between treatments (ANOVA; *P* = 0.938).

Indeed, *H. halys* adults have been successfully tracked using harmonic radar to establish retention times in, and movement from, trap crops (Blaauw et al. 2016) and attract-and-kill sites (Morrison et al. 2016). However, harmonic radar, while powerful for tracking individual insects, is not suitable if large numbers of individuals need to be tracked. For example, a model was developed for establishing plume reach and trapping area of pheromone-baited traps using large numbers of marked individuals (Miller et al. 2015). Recently, large cohorts of fluorescent-marked adult and nymph *H. halys* using marking methods reported here were released to determine plume reach and trapping area of pheromone-baited traps (Kirkpatrick et al. 2019a). Experiments of this type would not have been possible using certain other marking techniques such as harmonic radar due to the numbers of marked individuals needed and because harmonic radar equipment is often not available because of the expense for the equipment.

Marking with the fluorescent powder solution was quick and efficient allowing 50 insects to be marked within 5 min. In contrast, marking *H. halys* with neon markers is labor-intensive, taking approximately 1 h to mark 50 adults. However, persistence of marks is another consideration. *Halyomorpha halys* marked with neon markers were detectable for up to 2 wk after release. After 48 h, 1 wk, and 2 wk following release, we detected 17.1%, 2.6%, and 2.1% of *H. halys* that were released (Table 2). Higher detection occurred in soybean compared with woods; overall dispersal between soybean and woods was low (Table 2). We were able to detect adults and nymphs marked with fluorescent solutions for 1 wk following release (Kirkpatrick et al. 2019a). However, future semifield studies could be conducted to determine the exact time period the different types of marks persist on *H. halys*. Similarly, using the immunomarking technique, marked *H. halys* were recovered 1 wk after marking (Blaauw et al. 2016). In contrast, harmonic radar systems have limited detection time. Tagged adults and nymphs have been recovered from baited apple trees only as long as 24 h (Morrison et al. 2016) and 48 h, respectively, following release (Kirkpatrick et al. 2019b).

Table 2. Percent of marked adult *Halyomorpha halys* recaptured in soybean field and wooded area over time, showing crossover and dispersal into either the area they were released or to the nonrelease area

	Recaptured					
Area released	**48 h**		**1 wk**		**2 wk**	
	Soybean	**Woods**	**Soybean**	**Woods**	**Soybean**	**Woods**
Soybean	11%	1%	1%	1%	0.7%	0.5%
Woods	0.09%	5%	0.4%	0.2%	0%	0.9%

Marking *H. halys* adults and nymphs using fluorescent powders proved to be an inexpensive, fast, and simple method and is a recommended marking technique for future studies, especially when large numbers of individuals are required. Because fluorescent powders have been used previously for a range of insect orders, this method could easily be applied to new invasive insects to understand dispersal and movement capabilities when they arrive in the United States. As international trade expands, the probability of introduction of exotic species increases and more invaders will be discovered in the coming years. Furthermore, this technique can be used alone or in combination with other types of marking methods such as immunomarking, harmonic radar, and/or UAV studies to further examine dispersal behavior, plant–insect interactions, relationships with conspecifics, and movement throughout the landscape as plant phenology changes over the growing season.

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