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Evaluation of a fluorophore for marking navel orangeworm (Lepidoptera: Pyralidae)

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Navel orangeworm, *Amyelois transitella* (Walker), is a key pest in California's almonds, pistachios, and walnuts. This insect's strong dispersal capacity can potentially undermine the efficacy of localized management efforts. The timing and extent of *A. transitella* movement between orchards remain unclear, and more studies are needed to better characterize its landscape ecology. Mark-release-recapture studies offer a potential solution but require a reliable insect marker that is durable, easily identifiable and has minimal impacts on *A. transitella* longevity and flight ability. To address this, we evaluated 4 colors (red, blue, green, and yellow) of a fluorophore marker (SmartWater) for adult *A. transitella*. We conducted laboratory assays to assess moth flight ability and mortality, as well as marker persistence over time using both quantitative (plate reader) and qualitative (visual observation) fluorophore detection methods. Results demonstrated that none of the 4 colors negatively affected *A. transitella* flight ability or mortality. Green and yellow markers were persistent and readily identified by both detection methods, unlike blue and red markers. Although marker degradation was observed over time with the quantitative method, a high percentage of moths (70.3%) retained green and yellow markers after 14 days. In contrast, these markers did not show significant degradation using the qualitative method, with over 94.2% of moths showing fluorescence 14 days postmarking. These findings highlight the strong potential of green and yellow markers for field studies with *A. transitella*. We discuss their use in future mark-release-recapture studies and compare the 2 fluorophore detection methods.

Keywords: UV fluorescent marker, external insect marker, dispersal, tree nuts.

Introduction

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is a key pest of almonds, pistachios, and walnuts in California (Grant et al. 2020, Haviland et al. 2020a, 2020b). Adults deposit eggs directly onto new crop nuts, and the larvae that emerge feed on the developing kernel (Wade 1961, Michelbacher and Davis 2014). Not only does this reduce crop yield and quality, but infestation by *A. transitella* is associated with the presence of *Aspergillus* fungi (Palumbo et al. 2014) that produce aflatoxins, which are known human carcinogens heavily regulated in key markets. Currently, management of *A. transitella* includes crop sanitation, mating disruption, well-timed insecticide applications, and timely harvest (Wilson et al. 2020). However, with a strong dispersal capacity (Sappington and Burks 2014), *A. transitella* can move between orchards (Bayes et al. 2014), potentially undermining the efficacy of localized on-farm management efforts. Thus, more information is needed regarding the timing and extent of *A. transitella* movement between orchards to improve pest management strategies, and mark-release-recapture

studies are critical to this effort. A reliable external marker is essential for such studies.

Various insect markers and marking techniques have been explored to track insect movement in field settings. For instance, previous studies have examined the use of protein markers (Klick et al. 2014, Blaauw et al. 2016, Tait et al. 2018), isotopes (Lafleur et al. 1985, 1987, Lafleur and Hill 1987), and fluorescent dust (Stern and Mueller 1968, Byrne et al. 1996, Isaacs and Byrne 1998) for investigating insect activities in the field. Since no marker performs consistently across all insects, entomologists are continually seeking reliable markers and marking methods for their target species and field conditions (Hagler and Jackson 2001). In recent years, liquid colored fluorophores have gained attention for use as insect markers (Hagler et al. 2021, 2022, 2023).

These colored fluorophores have shown promising results as arthropod markers across various species. For instance, Faiman et al. (2021) reported that fluorophore markers did not lead to significantly lower survival or oviposition rates in *Anopheles gambiae* s.l.,

and remained detectable on marked mosquitoes throughout a 3-wk observation period. Additionally, research by Hagler et al. (2021) and Hagler et al. (2022) suggested that fluorophore markers could serve as effective markers for multiple insect species, such as *Lygus hesperus*, *Bemisia tabaci*, and *Chrysoperla* spp., with Hagler et al. (2024a) later reporting that the fluorophore markers did not affect *L. hesperus* roaming and flight speeds, distances, and durations. Similarly, Paul et al. (2024) reported that fluorophore markers persisted throughout the lifespan of the parasitoid *Trissolcus japonicus*, with marked individuals recovered up to 100 m from the release point, highlighting the markers' potential for dispersal studies.

Despite these encouraging findings for various insects, the applicability of fluorophores as external markers for lepidopterans has been unexplored until recently. A recent study on *Helicoverpa zea* identified 'Cartax Green' fluorophore as a potential marker for this lepidopteran species (Hagler et al. 2024b). Traditionally, common methods for marking lepidopterans include wing clipping (Klepetka and Gould 1996), dyes acquired through larval feeding (Ostlie et al. 1984, Hagler and Jackson 2001, Vilarinho et al. 2011), and fluorescent dusts (Adams et al. 2020). For *A. transitella*, previous work has demonstrated the use of Calco Red N-1700, an oil-soluble dye, for internal marking of adult moths (Andrews et al. 1980). However, this internal marker is obtained through larval feeding, which involves a lengthy acquisition process. While fluorescent dusts have occasionally been used for externally marking *A. transitella*, their effects on the moth have not yet been thoroughly investigated and documented. There is concern about using fluorescent dusts for marking *A. transitella*, as it has been found to have negative impacts on other species of Lepidoptera. For instance, marking with fluorescent dust impaired the ability of male painted apple moths, *Teia anartoides*, to detect pheromones and respond to calling virgin females (Stephens et al. 2008).

Given the current lack of a validated external marker for *A. transitella* and the promising results from previous fluorophore marker studies, we carried out a series of experiments to assess the feasibility of using fluorophores for marking adult *A. transitella*. We tested 4 fluorophore colors (red, blue, green, and yellow) to evaluate their effects on *A. transitella* flight ability and mortality. We also examined marker uptake and persistence using a plate reader, a quantitative method that measures the relative fluorescence intensity of specimens. Since previous research indicated that certain fluorophore colors were easily detected on some insect species via visual observation—a qualitative method with a simple setup (Hagler et al. 2021, 2022)—we selected the colors exhibiting strong persistence in the quantitative evaluations for qualitative assessments. In these qualitative evaluations, we shined a UV light on the specimens and visually inspected for the presence of fluorophores. Unlike the quantitative method, which measures relative fluorescence intensity, the qualitative method only detects the presence or absence of fluorophores but offers a simpler and more cost-effective approach. We compare these 2 fluorophore detection methods, discuss the use of fluorophore markers in future mark-release-recapture studies, and highlight how these markers could support an *A. transitella* sterile insect release (SIR) program, which has recently gained attention and shown promise for managing this pest (Wilson et al. 2020).

Materials and Methods

Test Insects
The *A. transitella* used in our studies were mass-reared and nonsterilized adults acquired from the USDA Animal and Plant Health Inspection Service (APHIS) rearing facility in Phoenix, AZ. Larvae were reared on an artificial diet that contained Calco Red N-1700 (Oil Red 2144, Royce Global, East Rutherford, NJ). This internal marker was not part of the study but was rather an artifact of the rearing processes employed at the APHIS facility. Approximately 10,000 chilled moths were packed and shipped in an insulated carrier box for overnight delivery to the University of California Kearney Agricultural Research and Extension Center (Parlier, CA), where we conducted the flight ability, mortality, and retention time studies. All delivered moths were used in the studies within 2 d of arrival and stored in a refrigerator at approximately 5 °C prior to use.

Study 1: Flight Ability
We investigated the effects of fluorophore markers, SmartWater (SmartTrace, DeterTech, Telford, England), on *A. transitella* flight ability. These water-based fluorescent solutions were initially designed for forensic marking in criminal investigations due to their unique property of being invisible under normal light (i.e., visible spectrum) and visible under UV light.

We began this study by creating an opening (≈ 2.5 cm dia.) on the side of a polypropylene cup (≈ 946.4 ml) with a lid and introducing 10 moths of the same sex into the cup. Subsequently, we inserted the mouthpiece of a nebulizer (Drive NEB KIT 500, Medical Depot., Inc., Port Washington, NY, USA) into the cup's opening and then connected the nebulizer to a flowmeter (3000 flowmeters, BROOKS Instrument, Hatfield, PA), which was attached to a standard laboratory air pump via a hose. The flowmeter measured the airflow rate generated by the air pump. This marking technique was adapted from Hagler et al. (2022). To mark the moths, we added 6.0 ml of the fluorescent solution or deionized water into the medication cup and turned on the air pump to 10 standard cubic feet per hour for 60 s. Following marking, we sealed the hole in the polypropylene cup with a foam plug and allowed the moths to air dry for 1 h before transferring them to flight cylinders for the flight ability investigation. Treatments in this study included 4 fluorophore colors: blue, green, red, and yellow, each tested at both high (100% fluorescent solution) and low (50% fluorescent solution) doses. Deionized water served as the control treatment and was used to dilute fluorescent solutions for low-dose treatments.

The flight cylinders were PVC coupling sockets (15.2 cm dia. × 16.0 cm height, Spears Manufacturing Company, Sylmar, CA) coated with Fluon (Insect-a-Slip, BioQuip Products, Inc., Rancho Dominguez, CA) on the inner surfaces, permitting moths to exit the cylinders solely through flight (based on Carpenter et al. 2012). We assessed moth flight ability and initiation by introducing 10 moths of the same sex that had received the same treatment into a flight cylinder and recording the number of moths flying out of the flight cylinder within 3 d. The flight cylinders were maintained at 28.7 °C, 40% RH, and 16:8 h L:D. This study followed a randomized complete block design with 6 replicates.

Study 2: Moth Mortality and Fluorophore Retention Time via Quantitative Evaluation
The fluorophore treatments tested in this study were consistent with those in the flight ability study. We placed 50 moths of the same sex in a polypropylene cup (≈946.36 ml) and marked them for 5 min using the same setup as in the flight ability study. After marking, we sealed the hole of the polypropylene cup with a foam plug, allowed the moths to air dry, and then immobilized them by chilling at 4 °C for 5 min. Subsequently, we transferred 40 moths from each cup to pop-up insect cages (30.5 × 30.5 × 30.5 cm, Shenzhen Tongzhou Technology Co., LTD, Shenzhen, China). Within each cage, 10

moths of the same sex that had received the same treatment were housed together, totaling 72 cages used. Water was provided ad libitum to the moths in each cage via a 50 ml uncapped centrifuge tube (Corning Incorporated, Corning, NY) filled with water, with cotton pads sealing the opening. The remaining moths (10 moths per cup) were retained in the cup, recorded for 0-d mortality, and placed in a freezer for later fluorophore examination. We hung the cages on pistachio trees in an orchard (≈0.8 ha; 36°35′26.3″N, 119°30′11.6″W) at the University of California Kearney Agricultural Research and Extension Center in Parlier, CA, with each cage randomly assigned to a pistachio tree, excluding trees on the orchard perimeter. The cages were left on the trees for various durations, including 1, 3, 7, and 14 d. Following each time interval, we retrieved 18 cages, each containing different combinations of moth sex and treatment, from the orchard for examination of the presence of fluorophores on the moths and for recording moth mortality. A moth was considered dead if it showed no movement when disturbed.

Prior to fluorophore examinations, we placed the moths individually into the wells of 48-well microplates (Costar 3548, Corning Incorporated, Corning, NY). To examine the presence of the fluorophore marker, we utilized a plate reader (Tecan Infinite M1000 Pro microplate reader, Tecan Trading AG, Switzerland) with specific excitation and emission wavelength settings for each fluorophore color (Table 1) to measure the relative fluorescence units (RFU) of each moth. We also measured the RFU of water-treated moths using the excitation and emission wavelengths for each fluorophore color. A moth was considered positive for the presence of fluorophore if its RFU exceeded the critical threshold value (CTV) of water-marked samples measured using the same wavelength settings. The CTV of water-marked samples was determined as the mean RFU plus 3 standard deviations of the water-marked samples (Hagler 1997, Hagler et al. 2022). This study was an A × B × C factorial design, where fluorophore treatment (9 levels: 4 colors with both high and low doses and a water treatment), sex (2 levels: male and female), and observation time (5 levels: 0, 1, 3, 7, and 14 days after treatment) were independent variables. Each combination of fluorophore treatment, sex, and observation time was replicated 9 times in total from 20 September to 30 November in 2022 (5 replicates) and 3 August to 12 October in 2023 (4 replicates). Throughout the study period, according to a data logger placed in the orchard, the average, minimum, and maximum temperatures were 14.0 °C, –0.5 °C, and 33.6 °C, respectively, in 2022, and 21.6 °C, 7.5 °C, and 35.9 °C, respectively, in 2023.

Study 3: Fluorophore Retention Time via Qualitative Evaluation

Based on the results from Study 2, we proceeded to investigate the retention time of the green and yellow markers through visual observation. To accomplish this, following the fluorophore examinations conducted with the plate reader in Study 2, we examined the same set of moth samples treated with water, green, and yellow colors for the presence of fluorophores using a handheld UV flashlight (TANK007

Table 1. Plate reader settings for each fluorophore color

Plate reader settings	Fluorophore color			
	Blue	Green	Red	Yellow
Excitation wavelength (nm)	365	365	365	365
Emission wavelength (nm)	430	535	630	577

UVE2, Shenzhen Grandoor Electronics Co., Ltd., Shenzhen, Guangdong Province, China). The examination involved directly inspecting each moth sample placed in the well of the microplate after removing the microplate lid, with the UV flashlight positioned approximately 5 cm above the microplate. We ensured proper safety measures by wearing UV protection glasses (NoCry, Las Vegas, NV) throughout the examination process. A sample was considered positive for the presence of fluorophore if the examiner observed the fluorophore marker on the sample (Fig. 1). An experienced observer examined all the samples throughout the process to ensure consistency in the evaluation.

Statistical Analysis

For Study 1, we compared the numbers of moths flying out of flight cylinders in different treatments using a Generalized Linear Mixed Model (PROC GLIMMIX) with a normal distribution and identity-link function in SAS (SAS 2020). The effect of markers (fluorophore treatments and water control) was treated as a fixed effect, while the block (replicate) was considered a random effect. Since it is unknown whether these fluorophores affect male and female *A. transitella* differently, and previous studies suggested that males and females likely differed in flight behavior (e.g., flight distance and duration) (Sappington and Burks 2014), we initiated statistical analyses by considering sex as a fixed effect. Given the significant effect of sex in this study, we conducted separate analyses for male and female moths. A significant fixed effect was followed by Tukey's HSD for multiple comparisons.

In Study 2, we determined the effects of markers, time (the duration of cages left in the orchard), and their interaction on moth mortality and the presence of fluorophores on the moths using PROC GLIMMIX with a beta distribution and logit-link function in SAS (SAS 2020). As the effect of sex on moth mortality and the presence of fluorophores on the moths was significant, we analyzed the data from different moth sexes separately. In this analysis, the effects of fluorophore treatment and time were treated as fixed effects, and the block (replicate) was considered a random effect. Based on the results of this initial analysis, the data were reanalyzed for only the green and yellow markers when we investigated the effect of markers and time on the presence of fluorophores on the moths. Significant fixed effects were followed by Tukey's HSD for multiple comparisons.

In Study 3, we examined the effects of the green and yellow markers, time (the duration of cages left in the orchard), and their interaction on the presence of fluorophores on the moths using PROC GLIMMIX with a beta distribution and logit-link function in SAS (SAS 2020). Fluorophore treatment and time were treated as fixed effects, while block (replicate) was considered a random effect. Since the effect of sex on the presence of fluorophores on the moths was not significant, it was also treated as a random effect.

Results

In Study 1, while the effect of markers was significant on flight ability in male moths ($F_{8,40}$ = 2.25, P = 0.044), none of the fluorophore treatments resulted in a significant reduction in the numbers of moths flying out of the flight cylinders compared to the water control (Fig. 2A). On the other hand, the effect of the marker was not significant in female moths ($F_{8,40}$ = 0.77, P = 0.63), indicating that no fluorophore treatments led to significantly lower numbers of flyers compared to the water control (Fig. 2B).
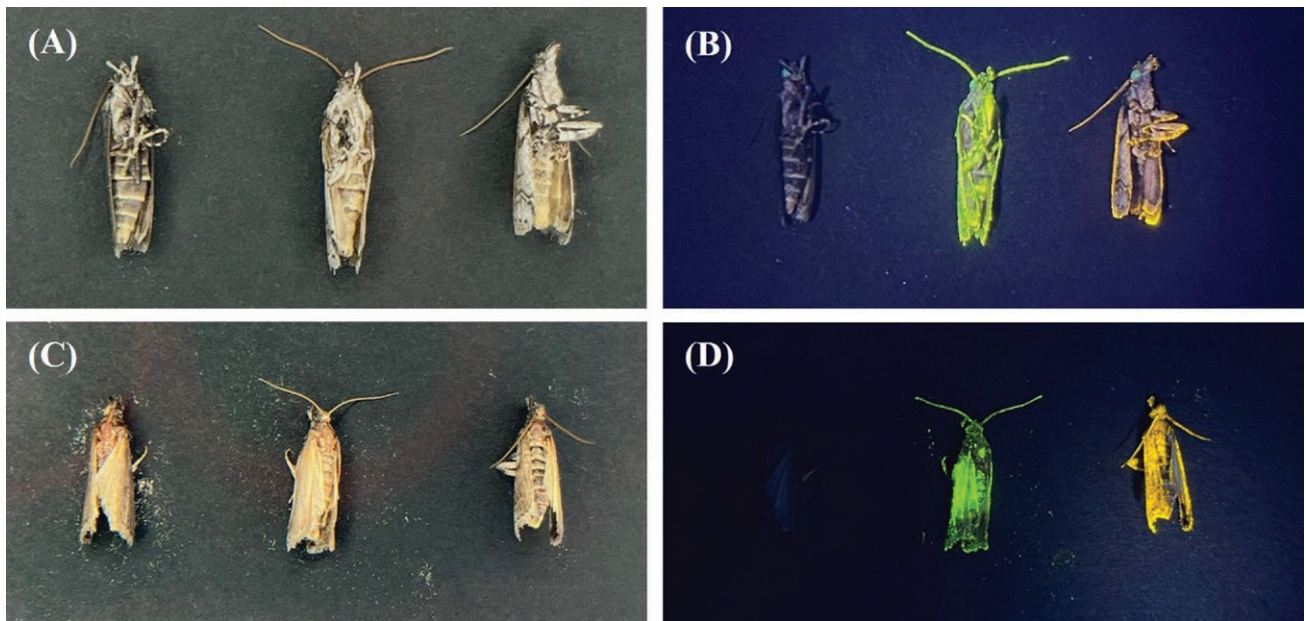
Fig. 1. Ventral view of water-marked (left), green-high-dose-marked (middle), and yellow-high-dose-marked (right) *Amyelois transitella* under normal light A) and ultraviolet light (365 nm) B); dorsal view of water-marked (left), green-high-dose-marked (middle), and yellow-high-dose-marked (right) moths under normal light C) and ultraviolet light D). Photos were taken approximately 1 h after marking.
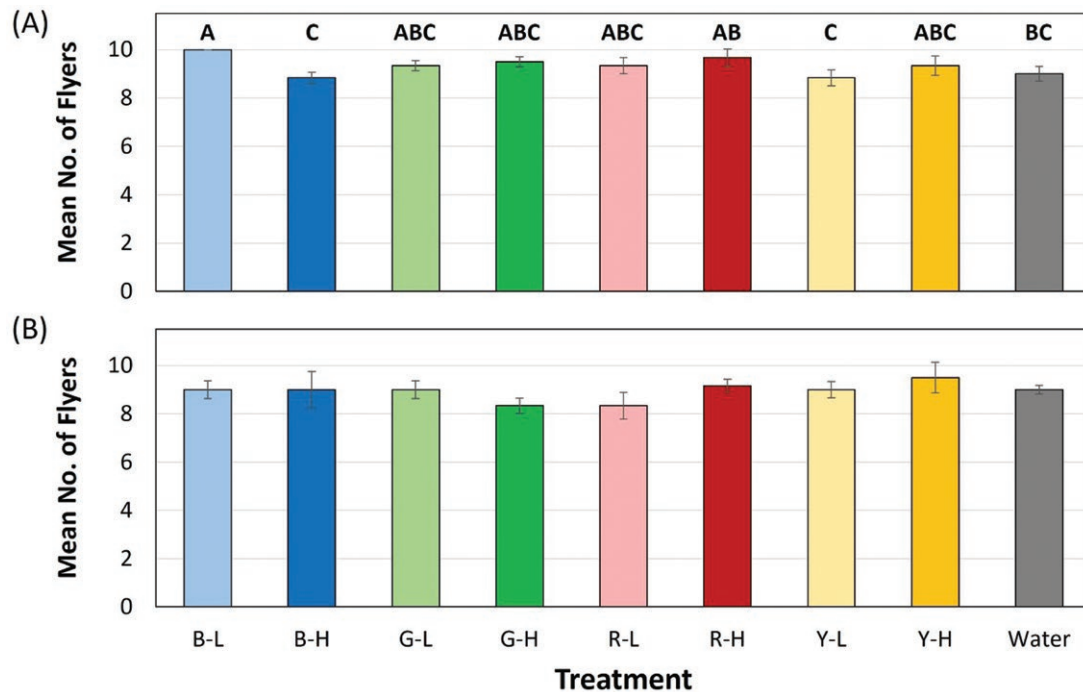

Fig. 2. Numbers (mean ± SE) of male A) and female B) *Amyelois transitella* flying out of the flight cylinders in different treatments (B-L = Blue low-dose, B-H = Blue high-dose, G-L = Green low-dose, G-H = Green high-dose, R-L = Red low-dose, R-H = Red high-dose, Y-L = Yellow low-dose, Y-H = Yellow high-dose). Bars with different letters above them indicate statistically significant differences (GLIMMIX; Tukey's HSD; $P < 0.05$). No significant differences among the treatments were observed in female moths (GLIMMIX; $P > 0.05$).

In Study 2, there was no significant difference in mortality between the fluorophore treatments and water control across any of the time intervals examined (male: $F_{8,352} = 0.64$, $P = 0.75$; female: $F_{8,352} = 0.40$, $P = 0.92$). There were, however, significant differences in mortality among time intervals (male: $F_{4,352} = 97.83$, $P < 0.001$; female: $F_{4,352} = 88.67$, $P < 0.001$). In both sexes, moth mortality significantly increased over time, with day 0 showing the lowest mortality (0 in both sexes) and day 14 showing the highest mortality (averaged at 53.8% and 59.6% in males and females, respectively) (Fig. 3). No significant interaction between marker and time was detected (male: $F_{32,352} = 0.75$, $P = 0.84$; female: $F_{32,352} = 0.49$, $P = 0.99$).
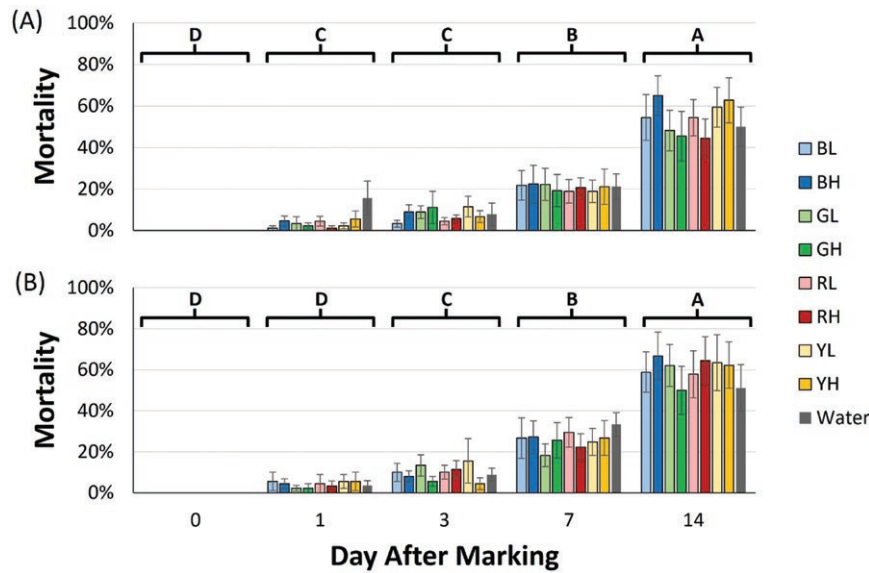
Fig. 3. Mortality (mean ± SE) of male A) and female B) *Amyelois transitella* on days 0, 1, 3, 7, and 14 after applications with different fluorophore color treatments (BL = Blue low-dose, BH = Blue high-dose, GL = Green low-dose, GH = Green high-dose, RL = Red low-dose, RH = Red high-dose, YL = Yellow low-dose, YH = Yellow high-dose). For each sex, moth mortality at observation times capped with different letters was significantly different (GLIMMIX; Tukey's HSD; $P < 0.05$).

Additionally, in Study 2, where we investigated the effects of fluorophore treatments on the presence of fluorophores on the moths, with all field exposure intervals pooled, significant fluorophore treatment effects were detected in both male and female moths (male: $F_{7,312} = 84.43$, $P < 0.001$; female: $F_{7,312} = 75.14$, $P < 0.001$). In male moths, the highest percentage of moths showing the presence of fluorophores occurred in high doses of colors green and yellow, followed by low doses of colors green and yellow, both low and high doses of color blue, and both low and high doses of color red, respectively (Fig. 4A). In female moths, the highest percentage of moths showed the presence of fluorophores in high doses of colors green and yellow and the low dose of color yellow, followed by the low dose of color green, both low and high doses of color blue, and both low and high doses of color red, respectively (Fig. 4B). All water-marked moths were identified as negative for fluorophores using the plate reader.

In Study 2, where we investigated the effects of time on the presence of fluorophores on the moths, excluding colors red and blue from our analyses, significant time effects were observed in both male and female moths (male: $F_{4,152} = 20.63$, $P < 0.001$; female: $F_{4,152} = 5.36$, $P = 0.001$), demonstrating a significant decrease in the presence of fluorophores on the moths over time. In male moths, the highest percentage of moths showing the presence of fluorophores occurred on day 0, followed by days 1 and 3, day 7, and day 14, respectively (Fig. 5A). In female moths, the highest percentage of moths showed the presence of fluorophores on days 0, 1, and 3, with no significant differences among them, followed by day 7 and day 14, respectively, where day 7 showed no significant differences with either day 3 or day 14 (Fig. 5B). Additionally, in this reanalysis, where colors red and blue were excluded, the effects of fluorophore markers were not significant in female moths ($F_{3,152} = 1.78$, $P = 0.15$) but were significant in male moths ($F_{3,152} = 5.09$, $P = 0.002$). In male moths, a significantly higher percentage of moths exhibited the presence of fluorophores in high-dose green and yellow treatments compared to their low-dose counterparts, with no significant differences between the colors within the same dose level. No significant interaction

between fluorophore treatment and time was observed in either sex (male: $F_{12,152} = 1.58$, $P = 0.10$; female: $F_{12,152} = 0.25$, $P = 0.99$).

In Study 3, where we examined moths using visual observation, the effects of the green and yellow markers ($F_{3,323} = 0.20$, $P = 0.90$), time ($F_{4,323} = 1.06$, $P = 0.38$), and their interaction ($F_{3,323} = 0.17$, $P = 0.99$) on the presence of fluorophores on the moths were not significant. Throughout the observation period, a high percentage of fluorophore-marked moths, ranging from an average of 94.2% (in the yellow low-dose treatment on day 14) to 100%, showed positive for fluorophores (Fig. 6). When comparing the results from the plate reader and visual observation, fluorophore-marked moths that were identified as positive by the plate reader were rarely identified as negative through visual observation (Supplementary Table S1). All water-marked moths were identified as negative for fluorophores through visual observation.

Discussion

Our findings indicated that the fluorophores did not cause adverse effects on *A. transitella* flight ability and did not lead to significantly higher moth mortality compared to the water control, which is consistent with previous studies by Hagler et al. (2021) and Rosser et al. (2022). Hagler et al. (2021) reported no significant impact of fluorophore markers on survivorship in *Hippodamia convergens* and *L. hesperus*, and Rosser et al. (2022) observed no adverse effects on the survivorship, fecundity, feeding behavior, and dispersal capacity of the predatory mite, *Phytoseiulus persimilis*.

In our investigation, we observed that fluorophore colors green and yellow outperformed red and blue, suggesting that the suitability of these fluorophores for insect marking may vary by color. This finding is consistent with research by Hagler et al. (2022), which revealed that the fluorophore "Cartex Green" was more detectable than "Magenta" and "Orange" when used on *H. convergens*. Although we noted a decrease in the presence of fluorophore marks on the moths over time using the quantitative method, a substantial proportion of moths still exhibited fluorophores 14 d after marking.
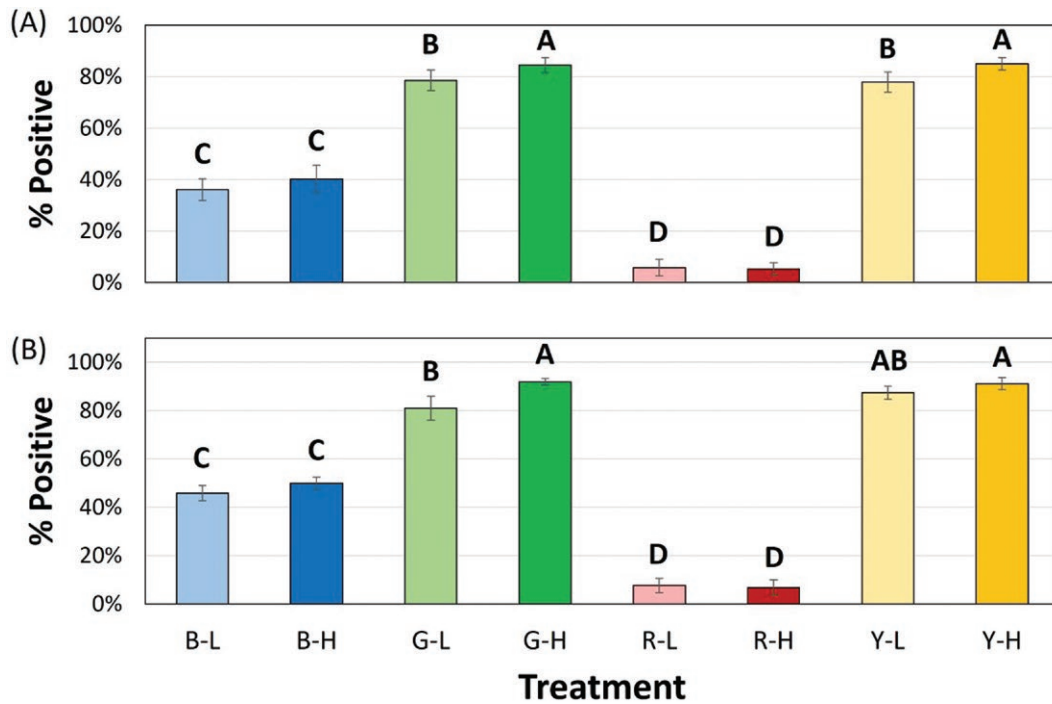
Fig. 4. Percentage of male A) and female B) *Amyelois transitella* (mean ± SE) (all field exposure intervals pooled) showing positive for the presence of fluorescence as detected by the plate reader in different fluorophore color treatments (B-L = Blue low-dose, B-H = Blue high-dose, G-L = Green low-dose, G-H = Green high-dose, R-L = Red low-dose, R-H = Red high-dose, Y-L = Yellow low-dose, Y-H = Yellow high-dose). For each sex, bars with different letters above them indicate statistically significant differences (GLIMMIX; Tukey's HSD; *P* < 0.05).
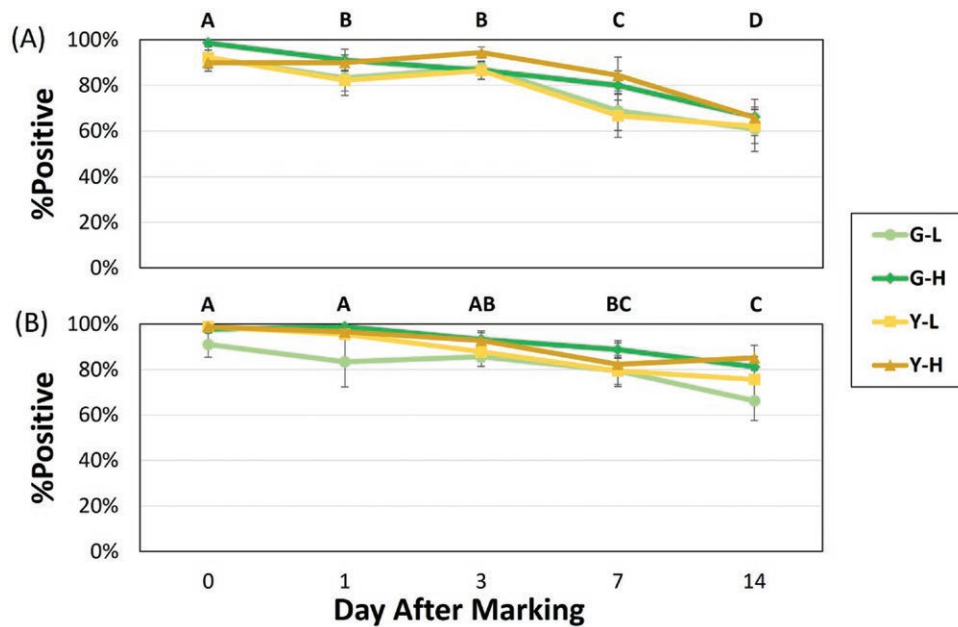
Fig. 5. Percentage of male A) and female B) *Amyelois transitella* showing positive for the presence of fluorescence as detected by the plate reader at different observation times (G-L = Green low-dose, G-H = Green high-dose, Y-L = Yellow low-dose, and Y-H = Yellow high-dose). The percentage of moths showing positive for the presence of fluorescence at observation times capped with different letters was significantly different (GLIMMIX; Tukey's HSD; *P* < 0.05).

In addition, through the qualitative method, we found that both low and high doses of colors green and yellow remained on the moths consistently, with 94.2% of the moths showing the presence of fluorophores 14 d after marking. The substantial persistence of fluorophores on the moths within a few days after marking makes them valuable tools for studying *A. transitella* behavior exhibited

within a short period after release, such as its mating behavior, since adult *A. transitella* typically mate and oviposit within 1 to 2 nights after emergence (Andrews et al. 1980, Wilson et al. 2020).

Regarding detection methods, visual observations for examining the presence of green and yellow fluorophores on the moths yielded more positive cases compared to using a plate reader. This outcome
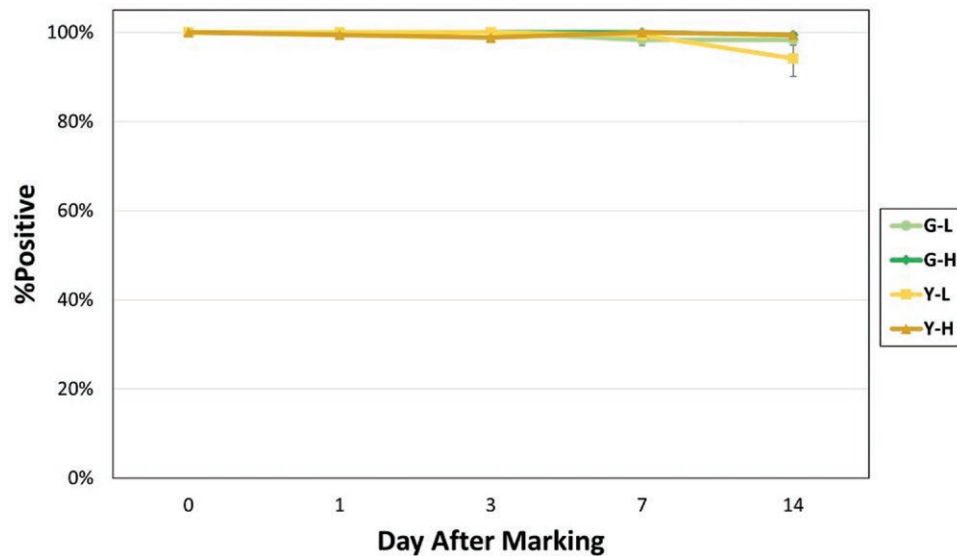
Fig. 6. Percentage of male A) and female B) *Amyelois transitella* showing positive for the presence of fluorescence as detected by visual observation at different observation times (G-L = Green low-dose, G-H = Green high-dose, Y-L = Yellow low-dose, and Y-H = Yellow high-dose). The effect of time (day after marking) was not significant (GLIMMIX; *P* > 0.05).

may be attributed to the high calculated CTV resulting from the high standard deviation among water-marked samples. Even with only slight marker degradation on the moths over time, where visual observation still identifies marked samples as positive, a high CTV resulting from the high standard deviation in RFU values makes it increasingly difficult to detect a marked sample as positive as time progresses. In our study, water-marked samples generally exhibited a low mean RFU value but sometimes had a high standard deviation when measured using the wavelength settings for the green fluorophore marker. Consequently, this scenario resulted in a high CTV, leading to some green-marked moths being scored negative for the presence of the fluorophore, despite being visually observed as positive. As the amount of fluorophore carried by the moths decreased, surpassing the high CTV became increasingly difficult. This can explain why, compared to the green-high-dose treatment, the green-low-dose treatment, where moths carried a lower amount of fluorophore, showed a lower percentage of marked moths scored positive for the presence of fluorescence from both examination methods across all observation times (e.g., on day 14, 57.9% in the green-low-dose treatment versus 70.4% in the green-high-dose treatment) (Supplementary Table S1). A similar occurrence of high standard deviation in RFU values was also noted in water-marked samples of *Mecaphesa celer* when assessed for the presence of the fluorophore "Cartax Green" (Hagler et al. 2022). The high variability of RFU in water-marked samples can be due to different degrees of autofluorescence in each individual sample. Another potential explanation for the increased number of positive cases in visual observations is that the plate reader assessed the samples directly from the top, whereas visual observers could examine the samples from various angles, facilitating a more thorough observation.

Previous research has found that visual observation was more reliable than the plate reader at detecting some fluorophore colors on certain insect species; however, it was less reliable in some other cases (Hagler et al. 2021, 2022). Overall, in our study, the plate reader yielded more conservative results compared to visual observation, and both fluorophore detection methods identified all water-marked moths as negative for fluorophores. In *A. transitella* mark-release-recapture studies, a reliable marker detection method should readily

detect and thus distinguish the marked and unmarked samples. That said, both the quantitative and qualitative methods can be used in *A. transitella* mark-release-recapture studies. However, in contrast to the quantitative method, visual observation requires a less expensive setup and can be less labor-intensive, as it does not involve the use of an expensive plate reader or the need to transfer the samples to the wells of microplates. Therefore, we recommend visual observation as a practical method for examining fluorophore green- and yellow-marked *A. transitella*.

Our research highlights the potential of using the green and yellow fluorophores as external markers for *A. transitella*. Findings from Hagler et al. (2024b) also demonstrated the promise of fluorophores for use in *H. zea*, suggesting their applicability for marking other lepidopterans. In both studies, the marking process achieved 100% marking success in treated moths. Compared to the commonly used internal marker, Calco Red, for lepidopterans, which has occasionally been reported as insufficient for detection due to moths not acquiring or retaining enough dye (Simmons et al. 2011), the fluorophores offer improved marking success. Regarding marker persistence, both Calco Red and the fluorophores perform excellently. Calco Red, once acquired, has been reported to be long-lasting (Hendricks and Graham 1970, Graham and Mangum 1971, Stephens et al. 2008). Similarly, the fluorophore "Cartax Green" was reported by Hagler et al. (2024b) to remain detectable on *H. zea* throughout the insect's average lifespan of 11 d, and in our study, the fluorophore presence on *A. transitella* remained high, with a slight decline, such that the lowest percentage of moths showing fluorescence was still above 94% by day 14.

In addition to the fluorophore markers' high marking success and excellent persistence, there are several other advantages to the use of fluorophores as external markers. First, detection of a marked moth can be achieved through nondestructive methods, unlike some internal markers (e.g., Calco Red), which may require insect dissection for marker examination. Second, fluorophore application is straightforward, and its versatility mitigates the need for extensive preplanning, as it is not acquired through the insect rearing process, unlike internal dyes, which rely on larval feeding for acquisition. Third, our findings indicated that 2 fluorophore

colors exhibited promise for marking *A. transitella*, making them valuable tools for studies requiring 2 external markers (e.g., comparing recapture rates from 2 different treatments concurrently in the same area). Although our studies focused on 4 specific fluorophore colors, there are additional fluorophore colors yet to be evaluated.

While using these fluorophores in mark-release-recapture studies could potentially support an *A. transitella* SIR program by tracking the behavior of released sterile insects, such as their spatial and temporal distribution in the field, which is crucial for determining the optimal release point and timing, prior to implementing these fluorophores as markers for SIR-based mark-release-recapture studies, additional research is needed to further evaluate their effects on other facets of *A. transitella* behavior. This includes various aspects of flight performance (e.g., flight distance), mating, and dispersal, as SIR depends on sterile insects successfully locating and mating with wild conspecifics to suppress field populations over time. Further studies should also investigate fluorophore degradation on a larger scale within the natural habitat of *A. transitella*, extending beyond relatively controlled environments (i.e., insect cages). Moreover, developing an efficient marking system that accommodates larger-scale applications will be crucial for supporting the *A. transitella* SIR mark-release-recapture research in field settings. One example of a mass-marking method is described by Hagler et al. (2024b), who found that directly submerging *H. zea* pupae in a fluorescent solution was a practical way to mass-mark adults. This method is intriguing and could potentially be adapted for use in the *A. transitella* system, offering a straightforward and efficient approach to mass-marking. However, during the mass-rearing process of *A. transitella*, larvae pupate in artificial diets, and pupae are encased within silk cocoons, which makes pupae collection challenging. Thus, applying this method to *A. transitella* requires further investigation.

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Supplementary material

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

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