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Evaluating *Drosophila suzukii* immunomarking for mark-capture research

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Abstract

Drosophila suzukii Matsumura (Diptera: Drosophilidae) utilizes 'Himalaya' blackberry, *Rubus armeniacus* Focke (Rosaceae), as a host and may invade berry and stone fruit crops from field margins containing this invasive weed. Laboratory and semi-field studies were conducted to determine (1) the persistence of protein marks including 10% chicken egg whites (egg albumin protein), 20% bovine milk (milk casein protein), and 20% soy milk (soy trypsin inhibitor protein) on topically sprayed *D. suzukii*, (2) protein retention on blackberry leaves, and (3) *D. suzukii* acquisition of protein after exposure to marked blackberry leaves for up to 14 days after application. All flies and leaves were assayed for the presence of the protein marks using protein-specific enzyme-linked immunosorbent assays. Egg albumin, milk casein, and soy trypsin proteins persisted on 94, 49, and 25% of the topically marked *D. suzukii*, respectively, throughout the 14-day study period. Egg albumin was retained on 100% of treated leaves for 14 days, regardless of environmental conditions. At least 50% of flies exposed residually to egg albumin-treated leaves were marked for 3 days, regardless of exposure time and environmental conditions. However, increasing fly exposure time to treated leaves in April and June appeared to improve protein mark acquisition. Acquisition of protein by flies from treated leaves for milk casein was inconsistent, and poor for soy trypsin, despite detectable levels on treated leaves. Egg albumin had the longest and most consistent persistence on flies, leaves, and flies exposed to leaves in laboratory and semi-field studies, under a variety of environmental conditions and exposure times.

Introduction

Spotted wing drosophila, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), is an invasive pest that is capable of causing major economic loss in berry and stone fruit crops. *Drosophila suzukii* is native to Southeast Asia and was first discovered in mainland USA, in California, in 2008 (Hauser et al., 2009; Walsh et al., 2011). *Drosophila suzukii* is now widely established across North America and Europe (Hauser, 2011; Calabria et al., 2012). In 2009, reports of yield losses in all susceptible crops ranged from negligible to 80% in Pacific coast states (Bolda et al., 2010). Adult flies oviposit in ripening fruit (Lee et al., 2011a). Fly larvae feed on the fruit interior, making the fruit unmarketable. Growers heavily rely on protective insecticide applications to manage this pest. A number of insecticides currently labeled for use in susceptible crops are efficacious against *D. suzukii* (Beers et al., 2011; Bruck et al., 2011); however, their increased use threatens existing integrated pest management programs and has high potential for insecticide resistance. Increased pesticide use also elevates the cost of production, but these expenditures are small in comparison to yield losses in the absence of management (Goodhue et al., 2011). A variety of cultivated and non-cultivated plant species serve as hosts (Lee et al., 2011b) ripening over an extended period of time (April–October) and may facilitate persistence and

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increased densities of *D. suzukii* throughout the Pacific states.

The most abundant invasive and noxious weed commonly found along field margins and riparian areas of commercial fruits throughout the Pacific Northwest of the USA is 'Himalaya' blackberry (HB), *Rubus armeniacus* Focke (Rosaceae) (Ringold et al., 2008). The effects of HB on *D. suzukii* population dynamics, presence, and movement between field margins and cultivated crops are unknown. Unpublished field observations from 2010 through 2013 suggest that *D. suzukii* populations increase in field margins containing HB and other non-cultivated hosts initially and subsequently colonize interiors of cultivated crops when fruit begins to ripen. Landscape ecology and overwintering studies from 2009 to 2013 also indicate that HB and diverse riparian habitat provide ideal refuge for *D. suzukii* adults (AJ Dreves, unpubl.). These observations suggest that the presence of HB in field margins is a key factor in annual *D. suzukii* infestations in neighboring crops.

An understanding of *D. suzukii* dispersal will aid in the timing of and need for pest treatments and may allow for implementation of alternative management strategies such as crop 'border-sprays', mass trapping, and possible future bait sprays to mitigate *D. suzukii* invasion, significantly reducing insecticide usage, potential negative environmental impacts, and grower expense. However, we currently lack definitive data on the timing and extent of *D. suzukii* dispersal from non-cultivated hosts neighboring cultivated plantings.

Insect mark-capture type dispersal research often requires (a) marker(s) to tag the resident insect population of interest. Ideal markers should not affect insect behavior (e.g., flight, growth, life span), withstand environmental degradation, be inexpensive and easy to apply over vast areas, and be easy to detect (Hagler & Jackson, 2001). Furthermore, the most appropriate marker may be species dependent. Therefore, studies are necessary to determine the most suitable mark for the mobile *D. suzukii* prior to conducting an extensive mark-capture study. Dust (Prasifka et al., 1999; Hagler et al., 2011) and dye (Schellhorn et al., 2004) markers have been used for mark-capture research, but they are difficult to apply over vast areas. Elemental markers (Prasifka et al., 2001; Qureshi et al., 2004) also have been used in mark-capture type research; however, high costs and potential for negative behavioral and developmental effects (Hagler & Jackson, 2001) likely limit the application of these markers in large-scale mark-capture studies for the small (2–3 mm) *D. suzukii*.

Protein marks have been shown to be cost effective, easy to apply, and easy to detect via enzyme-linked immunosorbent assays (ELISA) (Jones et al., 2006). Additionally, protein marks do not appear to have any adverse side effects on insects (Hagler, 1997; Slosky et al., 2012). Proteins have been used successfully in the field to study movement and dispersal of pests between managed and unmanaged areas of citrus (Boina et al., 2009; Krugner et al., 2012) and orchards (Jones et al., 2006; Horton et al., 2009; Basoalto et al., 2010). Currently, the efficacy of marking *D. suzukii* with proteins has not been determined. Also, little is known on the effect of environmental conditions (e.g., rain, temperature, humidity) on protein mark persistence.

The objective of this study was to identify the most compatible protein marker, which included egg albumin in chicken egg whites, milk casein in bovine milk, and soy trypsin inhibitor (hereafter referred to as soy trypsin) in soy milk, for future use in mark-capture dispersal studies of *D. suzukii*. The attributes of each mark type was determined by analyzing (1) protein persistence on topically sprayed *D. suzukii*; (2) retention on blackberry leaves under varying environmental conditions; and (3) residual acquisition of protein by flies exposed to marked blackberry leaves up to 14 days after contact. Suitable protein marks identified here will be used to study *D. suzukii* movement in agroecosystems.

Materials and methods

Laboratory colony

Drosophila suzukii originated from adults collected from infested fruit from grower fields in the Willamette Valley of Oregon in 2009. Male and female *D. suzukii* adults were placed in 75-ml plastic culture vials filled with ca. 9.4 cm^3 *Drosophila* cornmeal diet (San Diego Drosophila Stock Center, San Diego, CA, USA) with a light sprinkling of Fleisch-mann's active dry yeast (ACH Food Companies, Memphis, TN, USA) and capped with foam plugs. Cultures were maintained in climate chambers held at 22 ± 1 °C, 35 ± 5% r.h., and L16:D8 photoperiod. Field-collected *D. suzukii* were introduced into the colony on multiple occasions in 2010 and 2011 to ensure that the genetic make-up is representative of the field population. Adult flies, ranging from 3 to 15 days old, were used in the experiments.

Direct contact topical exposure

A laboratory study was conducted to evaluate the persistence of three protein marks that were topically applied to adult flies. The experiment was performed in a completely randomized block design with the date of protein application used as the blocking factor. Each protein treatment was replicated 3× on three application dates (4 May, 3 and 21 June 2011). An experimental unit of 50 *D. suzukii*

adults of each sex was randomly chosen from the colony and immobilized with a portable CO_2 dispenser (Genesee Scientific, San Diego, CA, USA) for 3–5 s, placed on a 100 × 15-mm Petri plate (VWR International, Randor, PA, USA), and treated with 2 ml of 10% chicken egg whites (vol/vol) (All Whites; Papetti Foods, Elizabeth, NJ, USA), 20% bovine milk (vol/vol) (Hy-Top 2% reduced fat milk; Federated Group, Arlington Heights, IL, USA), 20% soy milk (vol/vol) (WestSoy organic unsweetened soya milk; The Hain Celestial Group, Melville, NY, USA), or water (untreated control) using a Precision Potter Spray Tower (Burkard Scientific, Uxbridge, UK). Application treatments were randomly assigned to flies. Within an hour after treatment, treated flies were moved into respective transparent cages (22 × 22 × 27 cm) designated by treatment and replicate to avoid cross contamination, and maintained in climate chambers as previously described. The cages contained a Petri dish of the diet (as previously described) and a watering system (water container with sponge wick). To determine marker durability, five randomly selected *D. suzukii* of each sex were removed from each cage 1, 3, 7, 10, and 14 days after treatment, killed by freezing, individually placed into 1.5-ml microcentrifuge tubes, and stored at −80 °C until they were assayed for the presence of the protein mark by ELISA, as described below.

Indirect contact residual exposure

Residual exposure was defined as an unmarked fly's direct contact with a protein-marked leaf, i.e., a fly's acquisition of a given protein after walking over a protein-treated leaf surface. Protein mark acquisition and subsequent persistence was evaluated by exposing flies to treated blackberry leaf surfaces for 1, 10, or 60 min. Mark acquisition was performed as a semi-field experiment in a completely randomized block design with date of application (April, May, and June) as the blocking factor. Four mature cultivated blackberry plants were systematically selected from a stand of eight outdoor plants (buffer plant between each experimental treatment to avoid cross contamination) located at the USDA-ARS Horticultural Crops Research Unit (Corvallis, OR, USA). Plants were sprayed with fine droplets of chicken egg white, bovine milk, and soy milk, or water (control) using a hand-held spray bottle on 17 April, 4 May, and 21 June 2011 at the same concentrations as used above. Each plant was sprayed with a 500-ml solution of each treatment to ensure full coverage of all leaf surfaces at a time when no precipitation was forecasted for at least 24 h. Nine leaves were randomly selected throughout each plant canopy 1, 3, 7, 10, and 14 days after treatment, with measures to avoid contamination. Each leaf was trimmed to a 50-mm-diameter disc and placed into a 50 × 11-mm Petri dish, randomly assigned to 1, 10, or 60 min of exposure, and replicated 3×. Five *D. suzukii* adults of each sex were anesthetized with CO_2 for 3–5 s and placed into the Petri dish containing the treated leaf. After the exposure time elapsed, flies were killed by freezing, leaf samples were taken by pressing the end of a plastic drinking straw firmly into the surface and cutting a small section (fresh straws were used between samples to avoid cross contamination), and fly and leaf samples individually placed into 1.5 ml microcentrifuge tubes and stored at −80 °C until they were assayed for the presence of protein by ELISA.

ELISA analysis

Flies and leaves were analyzed using protein-specific ELISAs (Jones et al., 2006). In brief, each sample was soaked in 750 µl Tris buffered saline (pH 7.4) at 27 °C for 1 h at 100 r.p.m. on an orbital shaker and then assayed for the presence of protein using the anti-chicken egg albumin, anti-milk casein, or anti-soy trypsin inhibitor ELISA. Fly and leaf samples were scored positive for the presence of the protein mark if the ELISA optical density (OD) reading was three standard deviations greater than the mean negative control result (Hagler & Jones, 2010).

Weather station

Weather data were collected from iMETOS Ag weather stations (Pessl Instruments, Styria, Austria) with temperature/r.h. and leaf wetness sensors at a location within 4 km from the trial site (Table 1).

Statistical analysis

Protein on leaves and topically and residually treated flies were statistically analyzed using Proc GLIMMIX (SAS Ver. 9.3; SAS Institute, Cary, NC, USA), with ELISA OD as the continuous response variable and assuming Gaussian, exponential, or lognormal data distribution, where appropriate. Separate analyses were done for each protein (egg albumin, milk casein, or soy trypsin) from each study (leaf, topical, or residual contact). The predictor variables for 'leaf' were days and months, for 'direct contact' exposure time, sex, and days, and for 'residual contact' exposure time, sex, days, and month. Interaction terms were included as predictor variables, but only the highest-order interaction terms were analyzed as non-significant interactions were sequentially removed from the model. The model included month of application (April, May, and June) as a random factor in analysis of topically treated flies, but as a fixed effect in residual experiments because we were interested in the effect of month (i.e., environmental conditions). To detect ELISA OD differences across days, LSMeans comparison tests were applied.

Table 1 Mean (± SE) precipitation, air temperature, relative humidity, and leaf wetness during 2-week experiments performed in April, May, and June collected from iMetos Ag weather station within 4 km from the trial site

Month	Precipitation (mm)	Temperature (°C)			r.h. (%)	Leaf wetness (min)
		Mean	Min	Max		
April	4 ± 1.5	8 ± 0.3	2 ± 0.7	13 ± 0.6	76 ± 2.1	212 ± 68.1
May	2 ± 0.7	11 ± 0.3	6 ± 0.7	16 ± 0.7	76 ± 1.6	121 ± 37.8
June	0 ± 0.1	17 ± 0.4	10 ± 0.6	24 ± 0.8	71 ± 1.4	114 ± 45.1

Leaf wetness is the mean period when top and bottom leaf surfaces were wet.

To simplify, the LSMeans only compared means from each sex when the effect of sex was significant. Due to the variation by month, separate LSMeans tests were done for each month for the leaf and residual studies. To investigate the interaction between exposure time and day, the 15 combinations of exposure time and day (i.e., 1 min and 1 day) were compared altogether by LSMeans for each month of the residual study.

Results

Direct contact topical exposure

The egg albumin persisted on all *D. suzukii* for 7 days after the application and declined only slightly (<15%) after 10 and 14 days. The ELISA OD readings significantly declined over time and were higher for females than males (day effect: $F_{4,438} = 36.59$, $P<0.0001$; sex effect: $F_{1,438} = 7.40$, $P = 0.0068$; Figure 1A). Milk casein was detected on only 65% of the flies the day after the application and then decreased to 50% after 3 days, after which it remained relatively constant. The mean ELISA OD readings yielded by the milk casein-marked flies was almost 3× less than that of the egg albumin-marked flies (sex*day interaction: $F_{4,438} = 4.46$, $P = 0.0015$; Figure 1B). The persistence of soy trypsin on the flies sharply declined after the 1st day to less than 10% by day 14. ELISA OD readings for soy trypsin-marked *D. suzukii* were similar to the milk casein-marked individuals and gradually decreased over time. The females had significantly higher ELISA OD readings than males (day effect: $F_{4,435} = 36.46$, $P<0.0001$; sex effect: $F_{1,435} = 5.65$, $P = 0.018$).

Indirect contact residual exposure

Egg albumin was detected on 100% of the field-aged cultivated blackberry leaves over the course (14 days) of the study (Figure 2A, D, and G) despite exposure to a variety of environmental conditions (e.g., precipitation, temperature, relative humidity, and leaf wetness) during the three trial periods (Table 1: April, May, and June). Mean ELISA OD readings for leaves treated with chicken egg white in April, May, and June were high and showed very little variation over time (day effect, April: $F_{4,30} = 8.41$, $P<0.0001$; May: $F_{4,30} = 1.64$, $P = 0.19$; June: $F_{4,24} = 1.48$, $P = 0.24$; Figure 2A, D, and G). Milk casein was detected on ≥90% of all leaves over the 2-week study in April and May. However, in June the protein was not retained well on leaf surfaces. The percentage of leaves marked with bovine milk fluctuated for 10 days between 80 and 100% and then sharply declined to 30% by day 14. The milk casein ELISA OD readings were consistently lower than egg albumin and gradually declined over time (day effect, April: $F_{4,30} = 2.92$, $P = 0.038$; May: $F_{4,30} = 3.25$, $P = 0.025$; June: $F_{4,30} = 5.11$, $P = 0.0029$; Figure 2B, E, and H). Milk casein retention on leaves was lowest in June. The leaves treated with soy milk yielded similar ELISA OD values as the milk casein mark; that is, they were lower than egg albumin and lowest in June. In addition, ELISA OD readings for soy milk-treated leaves decreased over time (day effect, April: $F_{4,30} = 14.34$, $P<0.0001$; May: $F_{4,30} = 6.27$, $P = 0.0009$; June: $F_{4,30} = 7.65$, $P = 0.0002$; Figure 2C, F, and I). The total percentage of leaf samples containing soy trypsin was 100% in April; however, the retention of the mark on the leaves decreased to 60% after 2 weeks in May and June.

Exposure time*day interaction was significant for those flies exposed to the egg albumin treated leaves in all three trial periods (April: $F_{8,420} = 4.64$, $P<0.0001$, Figure 3A–C; May: $F_{8,420} = 15.14$, $P<0.0001$, Figure 3D–F; June: $F_{8,420} = 2.95$, $P = 0.0032$, Figure 3G–I). In June, female flies yielded significantly higher readings than males ($F_{1,420} = 5.60$, $P = 0.018$; Figure 3G, H, and I). Overall, at least 50% of the flies acquired and retained the egg albumin mark for 3 days after a 1-, 10-, or 60-min exposure period to the treated leaves (Figure 3). In April, the percentage of marked flies subjected to the 1- and 10-min exposure treatments sharply declined to less than 40% after 3 days (Figure 3A and B). However, 60% of the female flies subjected to the 60-min treatment acquired and retained the egg albumin for up to 10 days (Figure 3C). In May, ≥50% of the flies were marked over the duration of the

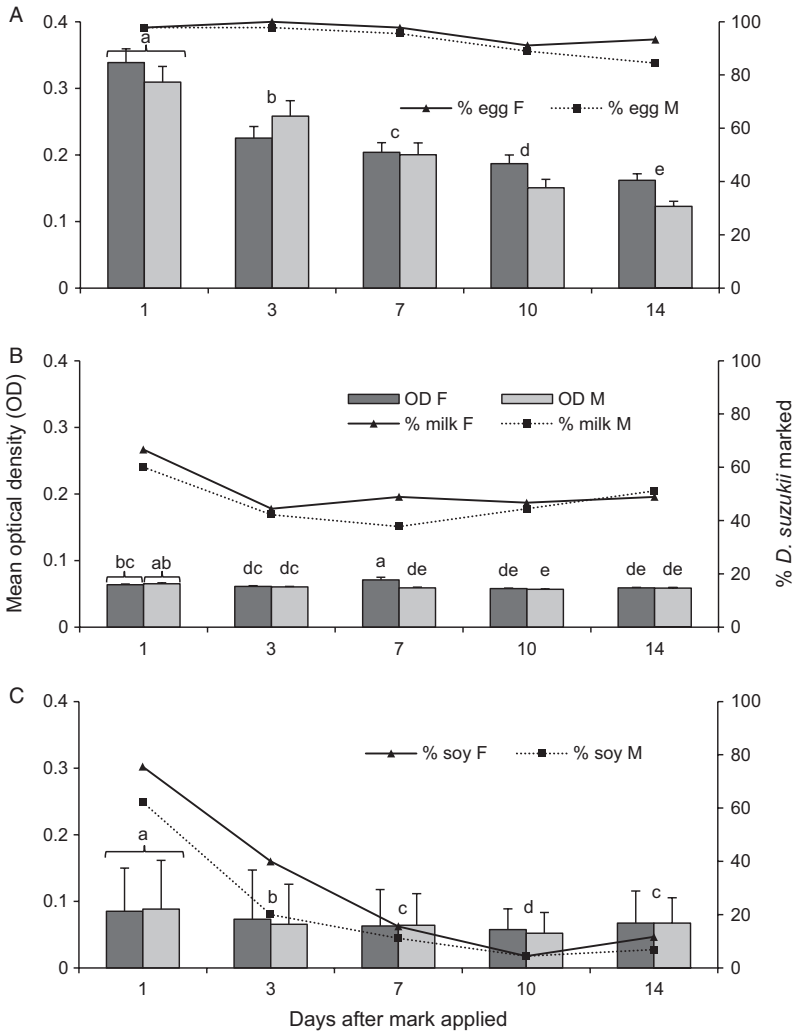

Figure 1 Effect of the number of days on mean (+ SE) ELISA optical density (OD) readings and % laboratory-marked *Drosophila suzukii* adult males (M) and females (F) treated topically with (A) egg albumin, (B) milk casein, and (C) soy trypsin proteins. Fly samples were scored positive for the presence of the protein mark if the OD reading was three standard deviations greater than the mean negative control result. Mean ODs within a panel (and in panel B within sex) capped with different letters are significantly different (LSMeans comparisons of transformed data: $P<0.05$). LSMeans estimates of soy trypsin-treated *D. suzukii* (C) were 2.50, 2.69, 2.78, 2.92, 2.82 for 1, 3, 7, 10, 14 days after treatment, respectively.

study and ≥80% for up to 10 days after residual exposure to the leaves (Figure 3D–F). In June, ≥50% of the flies were marked 7 days after exposure (Figure 3G–I). Increasing exposure time from 1 to 10 or 60 min resulted in more marked flies 10 and 14 days after contact exposure of the flies to the protein treated leaves.

There were no significant differences in the fly's acquisition from milk casein-treated leaves with regard to exposure time and day in the April and May experiments. However, in June, there was a significant exposure time*day interaction ($F_{8,420} = 11.58$, $P<0.0001$; Figure 4G–I) with males having significantly higher ELISA OD readings than females ($F_{1,420} = 11.74$, $P = 0.0007$). In April and May, less than 50% of the flies were marked throughout the 2-week study. In June, no flies were marked on day 1, regardless of exposure time (Figure 4G–I). Surprisingly, as the milk casein residue on the leaf aged, the percentage of marked flies increased to ≥60% after 14 days. High concentrations on the milk casein-treated leaves (e.g., high ELISA OD values) resulted in low ELISA OD readings and percentages of marked flies exposed to those leaves. Conversely, low ELISA OD readings on milk

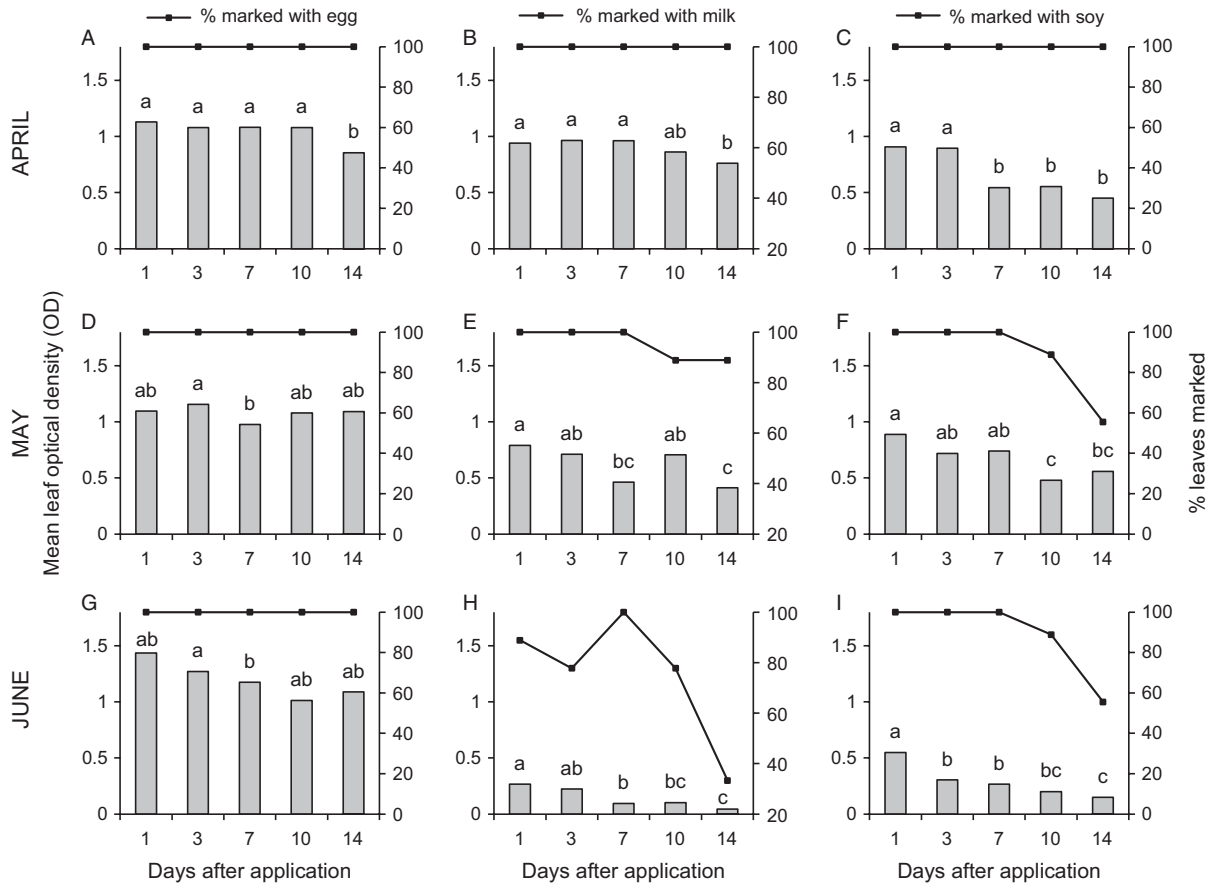

Figure 2 Effect of the number of days on mean (+ SE) ELISA optical density (OD) readings and % marked cultivated blackberry leaves treated in field in (A–C) April, (D–F) May, and (G–I) June with (A, D, G) egg albumin, (B, E, H) milk casein, and (C, F, I) soy trypsin protein. Leaf samples were scored positive for the presence of the protein mark if the OD reading was three standard deviations greater than the mean negative control result. Mean ODs within a panel capped with different letters are significantly different (LSMeans comparisons of transformed data: $P<0.05$).

casein-treated leaves resulted in higher readings and percentages of marked flies. This discrepancy is discussed below.

No differences were found in flies exposed to soy trypsin-treated leaves. However, in May and June, there were significant exposure time*day interactions (May: $F_{8,417} = 3.63$, $P = 0.0004$, Figure 5D–F; June: $F_{8,419} = 2.47$, $P = 0.013$, Figure 5G–I) and females had higher ELISA OD readings than males (May: $F_{1,417} = 6.24$, $P = 0.013$; June: $F_{1,419} = 11.12$, $P = 0.0009$). The percentage of flies marked containing soy trypsin was never greater than 20% in all trials.

Discussion

Throughout the 14-day laboratory trials, the vast majority (94%) of flies topically marked with egg albumin retained the mark, whereas only 49 and 25% of the milk casein and soy trypsin marked flies were positive for the mark, respectively. Female flies retained more egg albumin and soy trypsin than males. An interaction effect in milk casein did not permit analysis of sex alone. Females may have acquired more protein because of physiological (e.g., larger body size) (Hauser, 2011) or behavioral (e.g., feeding, grooming) differences. Regardless, quantifying female movement in the field is more important than male movement because females cause the direct damage to the fruit. Soy trypsin was readily detectable on the leaves, but not on flies exposed to those leaves. Specifically, the retention of soy trypsin on flies sharply declined after only 1 day. Similar findings have been reported by Jones et al. (2006). They suggested that the soy trypsin on leaves might flake off or dry, thus making the protein less available to the target insect.

Despite thorough coverage of egg albumin on leaf surfaces in April, May, and June, it appears that flies did not

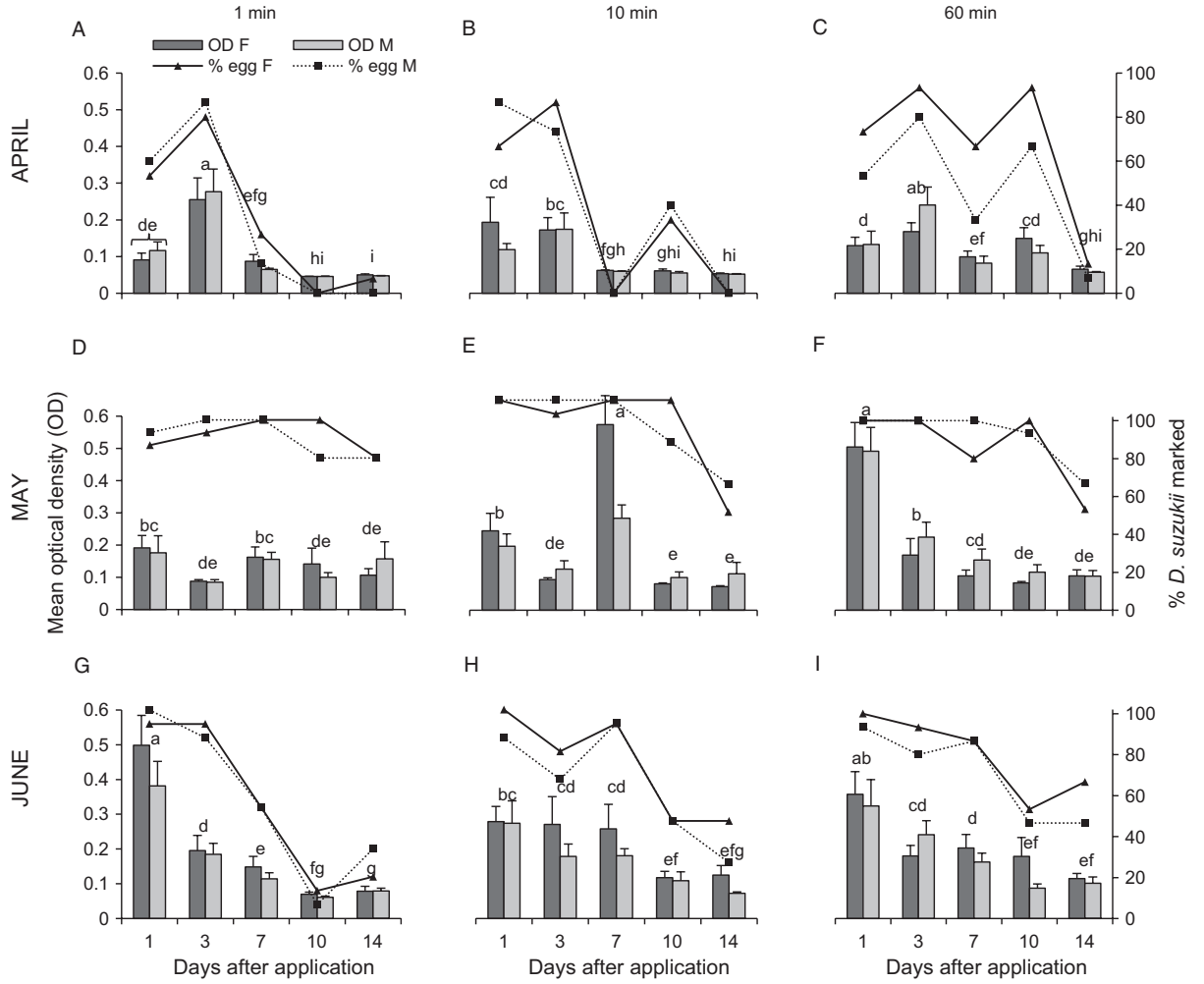

Figure 3 Effect of the number of days on mean (+ SE) ELISA optical density (OD) readings and % marked adult male (M) and female (F) *Drosophila suzukii* exposed to egg albumin protein-treated cultivated blackberry leaves for (A, D, G) 1 min, (B, E, H) 10 min, and (C, F, I) 60 min in (A–C) April, (D–F) May, and (G–I) June. Fly samples were scored positive for the presence of the protein mark if the ELISA OD reading was three standard deviations greater than the mean negative control result. Mean ODs within a month (i.e., group of three panels) capped with different letters are significantly different (LSMeans comparisons of transformed data: $P<0.05$).

acquire the egg albumin mark as well in the wettest (April) and driest (June) months of the study. Specifically, mark acquisition declined after 3 days in short exposure (1 and 10 min) and 7 days in longer exposure (60 min) treatments (except for a spike on day 10 in April); whereas in May, egg albumin mark acquisition only slightly declined after 10 days, regardless of the exposure time treatment. ELISA OD readings yielded by the flies exposed to egg albumin marked leaf tissue were similar for each exposure time treatment. Further research is needed to determine whether continuous light rain that occurred in May improved the acquisition of egg albumin from the plant tissue to the flies and to determine the effect of other weather conditions (i.e., heavy rain, dry periods) on fly marking.

Overall, the leaves treated with milk casein in June yielded poor ELISA results, but those flies exposed to the leaves yielded strong positive results (except flies exposed to day 1 leaves). This seems counterintuitive, as one might expect day 1 to have the highest percent flies marked. Instead, none of the flies were marked, suggesting that a protein mark interference with the ELISA (i.e., the samples may have had 'too much' milk casein on them) might have occurred (Hagler et al., 2011). If so, this phenomenon, known as 'steric inhibition' (Crowther, 2001) does not allow the target-specific antibody to bind to the antigen

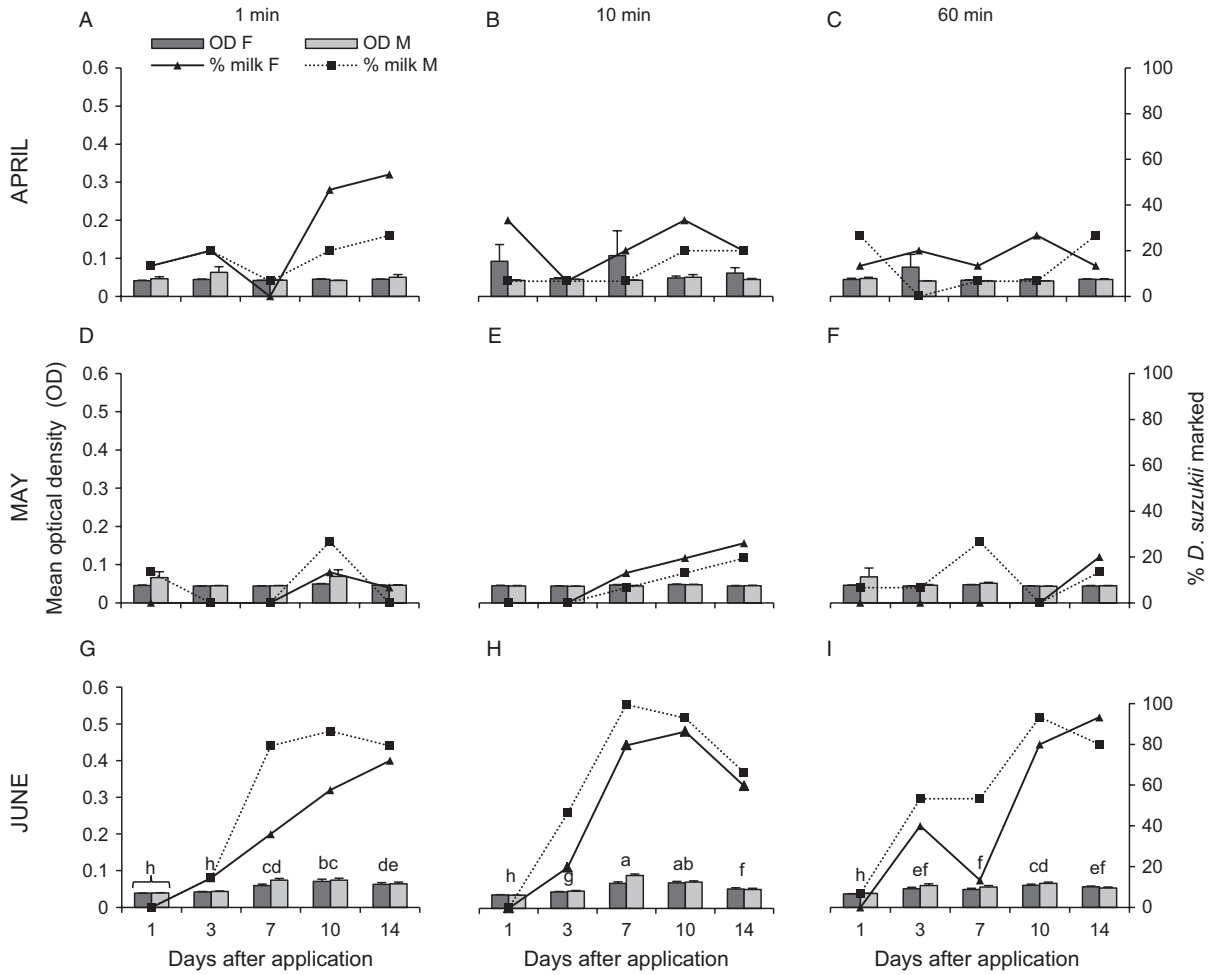

Figure 4 Effect of the number of days on mean (+ SE) ELISA optical density (OD) readings and % marked adult male (M) and female (F) *Drosophila suzukii* exposed to milk casein protein-treated cultivated blackberry leaves for (A, D, G) 1 min, (B, E, H) 10 min, and (C, F, I) 60 min in (A–C) April, (D–F) May, and (G–I) June. Fly samples were scored positive for the presence of the protein mark if the ELISA OD reading was three standard deviations greater than the mean negative control result. Mean ODs within a month (i.e., group of three panels) capped with different letters are significantly different (LSMeans comparisons of transformed data: $P<0.05$).

(protein), because the overcrowding of marker molecules interferes with antibody attachment. Therefore, it is possible that a more 'optimal' amount of milk casein was present on the leaves in June (compared to April and May) and improved as the protein degraded over time. This increasing trend in milk casein acquisition over time has been shown in previous research (Hagler & Jones, 2010; Irvin et al., 2012). For instance, milk casein-marked *Gonatocerus ashmeadi* Girault increased 12% from day 1 to day 11 (Irvin et al., 2012). None of these papers specifically address this increasing trend in percent marked, perhaps this was due to the fact that it was a more subtle trend than that observed in our study. Further research is needed to determine the 'optimal' amount of milk casein needed for effective acquisition. Milk casein may still be appropriate for large-scale field studies because applications will likely result in variable coverage (with some 'optimal' protein amount on leaves) for successful acquisition by flies (JR Hagler, unpubl.).

Many factors potentially influence the retention of proteins in field settings. For instance, abiotic factors such as rain, temperature, relative humidity, and dew point might influence protein retention on surfaces and protein acquisition by insects in the field. Jones et al. (2006) simulated rain events by washing treated leaf surfaces for various amounts of time. They found that milk casein had a greater rain-fastness than egg albumin or soy trypsin. However, a 1.5-mm rainfall event 12 days after applica-

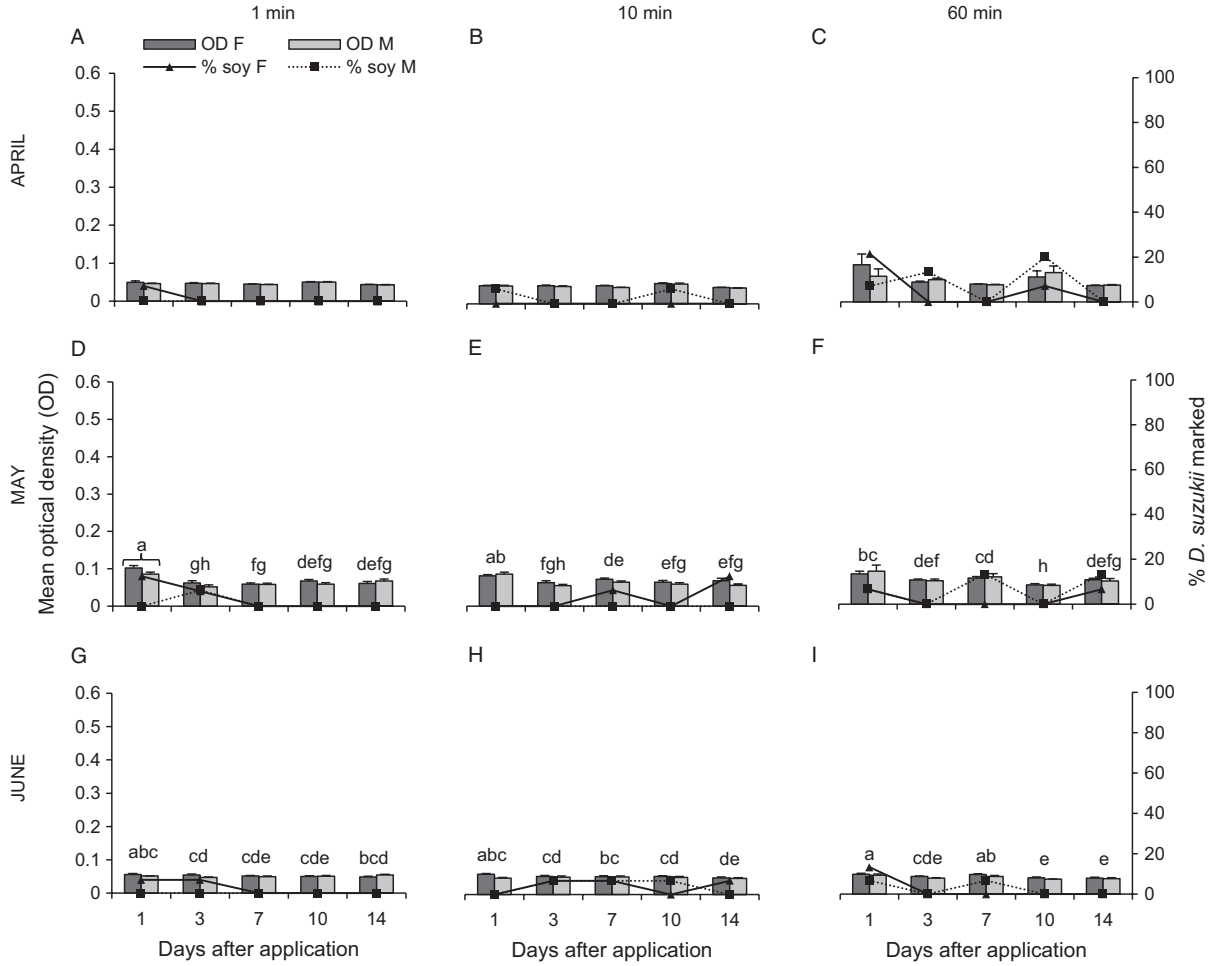

Figure 5 Effect of the number of days on mean (+ SE) ELISA optical density (OD) readings and % marked adult male (M) and female (F) *Drosophila suzukii* exposed to soy trypsin protein-treated cultivated blackberry leaves for (A–G) 1 min, (B–H) 10 min, and (C–I) 60 min in (A–C) April, (D–F) May, and (G–I) June. Fly samples were scored positive for the presence of the protein mark if the ELISA OD reading was three standard deviations greater than the mean negative control result. Mean ODs within a month (i.e., group of three panels) capped with different letters are significantly different (LSMeans comparisons of transformed data: $P<0.05$).

tion, in that same study, caused an increased immunoresponse to egg albumin and a decreased response to milk casein and soy trypsin to protein-treated leaf samples. Boina et al. (2009) showed that simulated rainfall to field-marked leaves decreased egg albumin, milk casein, and soy trypsin detection. Similarly, all proteins on field-aged leaves decreased in activity due to three rainfall events. Contrary to these previous studies, our data suggest that a 2-week mean of 4 (0–21.5) and 2 (0–8.2) mm rainfall per day in April and May, respectively, did not reduce protein detection on leaves. Furthermore, milk casein detection on leaves was poorest during the driest month, June (mean rainfall of 0.2 mm per day, ranging from 0 to 2 mm). The effect of temperature on proteins has also been investigated. Boina et al. (2009) showed that there was no significant difference in protein acquisition to insects exposed to treated leaves (residual acquisition) when held in 25 or 35 °C growth chambers. They concluded that temperature did not have an effect on protein retention. Our study shows that perhaps a combination of factors (e.g., rainfall, temperature) may influence protein detection. For example, the highest number of flies retained their mark with egg albumin after exposure to field-aged leaves during May (i.e., light steady rain and a mean temperature of 11 °C) compared to April (i.e., occasional heavy rain and cool weather) and June (i.e., dry and warm weather). Biotic factors related to insects and plants may also influence protein acquisition and retention. These factors include insect body size, body type (e.g., hairy, smooth, scaly), and behavior (e.g., feeding, grooming).

Hagler & Jones (2010) showed that *Hippodamia convergens* Guérin-Méneville and *Lygus hesperus* Knight obtained an egg albumin mark after only 5 min of contact exposure to protein-treated cotton leaf tissue; however, acquisition of the protein by *Trichoplusia ni* (Hübner) took between 20 and 240 min. Various additives incorporated into protein solutions may also influence protein detection; however, their effects remain uncertain (Jones et al., 2006; Williams et al., 2013). The water softener, EDTA, when added to protein solutions resulted in a positive effect (Jones et al., 2006) or no effect (Boina et al., 2009). These differences may have been influenced by abiotic (e.g., UV light, r.h.) and/or biotic (e.g., phenotypic traits of plants and insects) factors that contribute to the dissipation of protein-marked leaf surfaces and residually marked flies. For example, protein persistence on pubescent (apple leaves used in Jones et al., 2006) vs. glossy (citrus leaves used in Boina et al., 2009) leaf surfaces has not been tested. The biological solvent, dimethyl sulfoxide, when added to the protein solutions enhanced rabbit and chicken IgG protein retention for marking a beetle and its predator, but may have had a deterrent effect on beetles (Williams et al., 2013). Future studies are needed to test the effect of these factors on protein mark persistence and insect behavior in the environment.

We hypothesized that longer exposure time would improve protein mark acquisition by flies exposed to protein marked leaves. Increasing the exposure time of *D. suzukii* to treated leaf surfaces appeared to improve protein mark acquisition. We tested 1, 10, and 60-min exposure periods of insects to marked leaf tissue to mimic short, medium, and long field exposure situations. However, at present, it is unknown how much time *D. suzukii* spends in field margins containing 'Himalaya' blackberry. Another difference between these laboratory/semi-field studies and field-scale studies is the application method. Herein, we used a hand-held sprayer, whereas conventional spray equipment (e.g., airblast, boom, backpack, aerial spray) has been used to apply these proteins in the field (Krugner et al., 2012; Sivakoff et al., 2012; Swezey et al., 2013). Coverage will likely be dependent on the method of application.

In conclusion, the egg albumin protein appears to be the most effective mark for tagging *D. suzukii*. It was well retained on flies and leaves and is rapidly and readily acquired by flies exposed to protein-marked plant tissue. Rainfall did not appear to adversely affect egg albumin retention on the leaves; however, a steady amount of rain throughout the trial appeared to improve the acquisition of the protein by residual contact with protein-treated plants. Based on the results from this study, we have selected the egg albumin mark to tag the resident *D. suzukii* population for future mark-capture field studies.

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