Measuring natural enemy dispersal from cover crops in a California vineyard

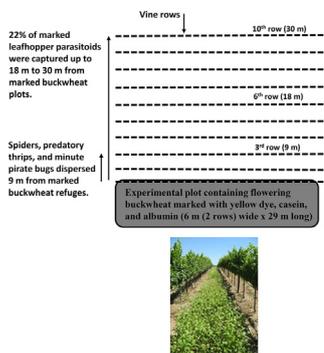N.A. Irvin[a,*], J.R. Hagler[b], M.S. Hoddle[a,c]

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GRAPHICAL ABSTRACT

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ABSTRACT

Dispersal of natural enemies from buckwheat cover crop plots embedded within a southern California vineyard during spring and summer was investigated by using an arthropod mark-capture technique. Specifically, arthropods were marked in flowering buckwheat plots by spraying plants with a "triple mark" solution containing yellow dye, casein protein, and albumin protein. In turn, we recorded the abundance of marked and unmarked natural enemies at a gradient of distances from the treated buckwheat plots into the vineyard. Natural enemies marked with yellow dye were identified visually, while the presence of casein and albumin protein marks were detected using anti-casein and anti-albumin enzyme-linked immunosorbent assays (ELISA). The percentage of natural enemies marked with yellow dye indicated that spiders, predatory thrips (Aeolothripidae), and minute pirate bugs (Anthocoridae) dispersed 9 m (i.e., 3 rows) from marked buckwheat refuges over a six day period. The percentage of leafhopper parasitoids (Anagrus erythroneurae S. Trjapitzin and Chiappini) marked with yellow dye indicated that 22% of marked parasitoids were captured up to 18 m (i.e., six rows) to 30 m (i.e., 10 rows) from buckwheat plots up to six days after marks were applied to cover crops. Up to 17% of natural enemies marked with yellow dye, albumin, or casein were captured in non-treated control plots, suggesting that parasitoids, spiders, minute pirate bugs and predatory thrips were able to cross the 36 m buffer zones used to separate marked buckwheat plots and unmarked control plots. Results comparing the percentage of parasitoids and 'other beneficials' marked with a double mark (where any two of the three marks were detected) between distances in buckwheat plots indicated that double marked parasitoids were found up to 30 m (i.e., 10 rows) from buckwheat refuges, while no double marked parasitoids were captured in control plots. No triple marked arthropods were captured. To exploit the dispersal capabilities of natural enemies, these results suggest that buckwheat refuges planted in California vineyards could be planted every 6th (i.e., 18 m) or 10th (30 m) row to gain potential benefits from providing natural enemies with flowering buckwheat refuges.

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1. Introduction

Floral and extrafloral nectar can maximize the longevity, fecundity, searching activity, and attack rates of natural enemies, and an increase in female-biased sex ratios of progeny of parasitoids and predators may result from access to these resources (Berndt and Wratten, 2005, Kost and Heil, 2005, Irvin et al., 2006, Hogg et al., 2011). Carbohydrate sources are important as adult parasitoids need to locate food at least once a day to avoid starvation (Azzouz et al., 2004, Idris and Grafius, 1995, Siekmann et al., 2001). Searching for food resources and hosts/prey involves metabolic costs and natural enemies need to minimize foraging time for food if reproductive success is to be maximized (Lewis et al., 1998). The time that natural enemies spend looking for carbohydrate resources in crops can be reduced by deliberately providing floral subsidies in the form of nectar and pollen (Wilkinson and Landis, 2005). Nectar can be provided to natural enemies in vineyards by sowing flowering plants as a cover crop or by tolerating flowering weed species (Barbosa, 1998). Cover crops also help maintain soil quality and contribute to erosion prevention, and their use is encouraged by the Californian wine industry which promotes sustainable practices through the Code of Sustainable Winegrowing Workbook (CSWW) (Dlott et al., 2002). The purported benefits that arise from the provisionment of cover crops that act as food sources for natural enemies in agroecosystems is a key component of conservation biological control (Gurr et al., 2004).

The use of buckwheat, *Fagopyrum esculentum* Moench, as a cover crop has been evaluated in vineyards in New Zealand (Berndt et al., 2002), Australia (Simpson et al., 2011) and California (Irvin et al., 2016), and is recommended as a cover crop plant for enhancing natural enemies in crops grown in arid soils in the southwestern USA (Grasswitz, 2013). Buckwheat can enhance natural enemy reproduction which may concomitantly reduce pest densities (Nicholls et al., 2000; Berndt et al., 2002, English-Loeb et al., 2003, Irvin et al., 2014). Other attributes favoring the selection of buckwheat as a cover crop are inexpensive seed that is readily available and germinates easily, short sowing to flowering times, and tolerance of poor growing conditions (Angus et al., 1982, Bowie et al., 1995, Grasswitz, 2013).

Natural enemies that utilize cover crops can disperse into adjacent crops and provide varying levels of pest control (Powell, 1986, Lővei et al., 1993, Freeman-Long et al., 1998). Despite potential benefits, habitat diversification through cover crop plantings in some instances may impede natural enemy movement and host/prey location efficiency (Sheehan, 1986, Frampton et al., 1995, Mauremootoo et al., 1995). Further, cover crops may act as 'sinks' for some species of natural enemies, which negatively affects pest suppression (MacLeod, 1999). To determine whether natural enemies will disperse from a cover crop into a high value crop it is important to determine the distances over which natural enemies will move (Gurr et al., 2005, Wratten et al., 2007). Consequently, understanding natural enemy dispersal dynamics from cover crops helps determine the size and spacing of cover crop patches in cropping systems (Landis et al., 2000).

Effective arthropod marking and tracking techniques are essential for evaluating the movement of natural enemies in an agroecosystem (Lavandero et al., 2004). Hagler et al. (1992, 2002) first described and applied mark-release-recapture methods to mark arthropods with foreign proteins (e.g., vertebrate IgGs). In turn, the protein marks were detected on field-collected specimens using anti-protein specific enzyme-linked immunosorbent assays (ELISA). Over a decade ago, an effective mark-capture method was described for marking arthropods directly in the field using inexpensive food proteins (e.g., chicken egg albumin, bovine milk, soy milk) with standard spray equipment (Jones et al., 2006). The ELISAs used to detect these food products are simple, inexpensive, sensitive, and have been standardized for large-scale processing (Hagler & Jones, 2010, Hagler et al., 2014). Irvin et al. (2012) demonstrated the potential of using albumin and casein proteins in combination with a fluorescent dye (a triple mark) to mark

Cosmocomoidea (formerly *Gonatocerus*) *ashmeadi* (Girault) (Hymenoptera: Mymaridae [Huber, 2015]), an egg parasitoid of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae). A double- or triple-marking system has the potential to reduce the rate of false positives that occurs using a single mark and this occurs when some insects are incorrectly identified as being marked when they are not (Irvin et al., 2012).

Here, we investigated the dispersal of natural enemies from buckwheat cover crop plots into surrounding grape vines by spraying flowering buckwheat plants with a triple mark containing yellow dye, casein, and albumin. The goal was to determine what types of natural enemies disperse from cover crops, and the distances over which they move. Beneficial insects that may be present in vineyards and enhanced through nectar cover cropping include parasitoids (e.g., *Gonatocerus* spp., parasitoids of sharpshooter eggs, and *Anagrus erythroneurae* Triapitzyn and Chiappini, a parasitoid of leafhopper eggs; both are mymarids) and generalist predators (e.g., anthocorids, coccinellids, chrysopoids and arachnids) (Van Driesche et al., 2008, Irvin et al., 2014). Minute pirate bugs (Anthocoridae) are generalist predators of thrips, spider mites, psyllids, mealybugs, aphids, white flies, insect eggs, and small caterpillars (Daane et al., 2008, Patterson and Ramirez, 2017). Predatory mites and thrips are the most significant predators of spider mites on grapevines (Hanna et al., 1997). Key pests of grapes in California include leafhoppers (Hemiptera: Cicadellidae), mites (Acari: Tetranychidae) and thrips (Thysanoptera: Thripidae) (CSWA, Wine Institute, and CAWG, 2012). Sharpshooters (Hemiptera: Cicadellidae) are significant pests of grape in California due to their ability to vector *Xylella fastidiosa* Wells et al., a xylem-dwelling plant pathogenic bacterium that causes Pierce's disease, a lethal malady of grapes (Blua et al., 1999). Other herbivore pests such as honeydew producing hemipterans like mealybugs (Pseudococcidae), psyllids (Psyllidae) and aphids (Aphididae) can be pestiferous in vineyards (Bettiga, 2013), especially if they develop mutualisms with ants which disrupt biological control (Navarrete et al., 2013, Schall and Hoddle, 2017). Information on natural enemy dispersal would enable optimization of cover crop plantings for conservation biological control of key grape pests in commercial vineyards in southern California.

2. Methods

2.1. Experimental design

In 2008, thirteen plots (28.7 m × 4.8 m [2 rows] separated by at least 36 m) were selected in four vineyard blocks of Cabernet Sauvignon grapes in a commercial organic vineyard in Temecula, CA, USA (GPS coordinates: 33° 3′26.18″N x 117° 00′52.12″W; elevation: 1637 feet). One or two buckwheat plots and control plots (vineyard plots that did not contain buckwheat) were randomly allocated per block, for a total of seven buckwheat and six control plots. The control plots were maintained according to vineyard management practices, which comprised of machine and hand cultivation between rows to remove unwanted weed vegetation. On May 1, 2008, buckwheat seed (Outsidepride, Salem, OR) was sown at recommended agricultural sowing rates, which translated to 336 g of buckwheat seed per 28.7 m plot, on a randomly allocated side of the row of each buckwheat plot. The other side of the row in the buckwheat plots was cultivated and sown with buckwheat on June 11, 2008. Buckwheat sowing was staggered to increase the length of time flowers were available for natural enemies. Buckwheat seed was re-sown in buckwheat plots 2–3 times between late May and mid-July 2008 at approximately 4 w intervals.

Sprinkler irrigation was installed on existing irrigation lines (drip irrigation for the vines which is common in southern California vineyards) to provide water to the buckwheat plots. Irrigation consisted of 5 sprinklers (blue Micro Bird Spinner sprinkler heads per plot, 45 L/h, 360° × 3.66 m diameter coverage; Temecula Valley Piping and Supply, Temecula, CA) each attached to 7 mm tubing which was supported by

an 18 cm bamboo stake on each side of the plot. Because buckwheat plots encompassed two rows, a total of 10 sprinklers per plot were installed. Each buckwheat plot was irrigated for 2 h the day after each sowing to promote germination, then approximately every 7–10 days for approximately 6 h. In addition to irrigation provided by vine irrigation schedules, buckwheat plots received supplemental irrigation with 60.5 L of water per plot, applied via a 60.5 L NorthStar ATV Tree Sprayer (Northern Tool + Equipment, Burnsville, MN) and 4WD motorbike (Kawasaki ATV), approximately three times a week over the period May to August 2008. Three out of seven buckwheat plots established and resulted in flowering buckwheat plants. Four assigned buckwheat plots that did not establish buckwheat were not subsequently used. Four control plots that were associated within the same blocks as the three established buckwheat plots were used. A site map detailing block and plot allocation and additional information on

factors affecting buckwheat establishment, plot maintenance, and quantity of water required to maintain the buckwheat cover crop is available (Irvin et al., 2016).

2.2. Application of marks

On July 22, 2008, buckwheat plants in each plot were sprayed with a total of 5 L of a triple marking solution with a Stihl backpack sprayer (Andreas Stihl AG & Co., Virginia Beach, VA). The triple marking solution consisted of 20% chicken egg white (strained All Whites, Papetti Foods, Elizabeth, NJ) (containing ~5% albumin [Anon 1, 2011]), 78% milk (Ralphs 2% Reduced Fat Milk, Inter-American Products, Inc., Cincinnati, OH) (containing ~80% casein [Anon 2, 2011]), and 2% yellow SARDI fluorescent pigment applied as a liquid dye in water (Topline Paint, Pty Ltd, Adelaide, SA, Australia). The four control plots
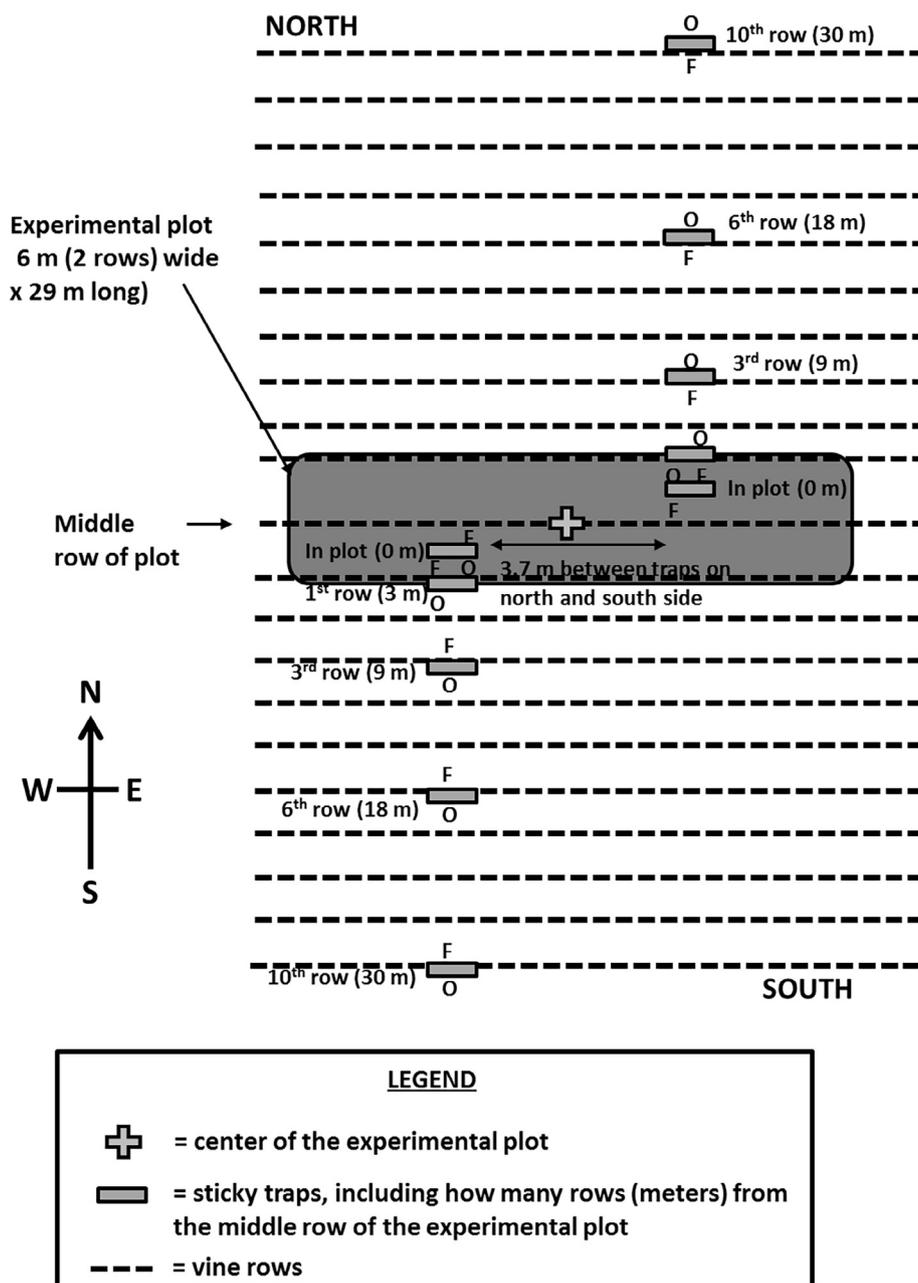

Fig. 1. Experimental design showing trap placement with a gradient of distances from the middle of an experimental plot (shaded region indicates the area of the buckwheat treatment or controls) in a north and south direction. Each trap was oriented parallel to vines and had an 'open side' (O) facing the plot row and a 'foliage side' (F) positioned towards grape vines.

were not treated with the triple mark. These plots were used to investigate the natural gradient of unmarked natural enemies within control plots, and to ascertain the effectiveness of > 36 m buffer zones used to separate treatments.

2.3. Arthropod monitoring

Transparent sticky traps (16.7 cm × 13.2 cm) were made from clear Perspex (Plaskolite Inc, Columbus, OH) coated on both sides with Tanglefoot (The Tanglefoot Company, Grand Rapids, MI). Transparent traps were used instead of colored sticky traps to avoid biasing trap catches (Horton, 1993, Takasu and Lewis, 1995, Hickman et al., 2001, Lavandero et al., 2005). Traps were first deployed on July 22, 2008 approximately 1 h after the application of the triple mark solution. Traps were collected and replaced with new traps on July 25, 2008. The replacement traps were collected on July 28, 2008. A diagram of an experimental plot is given in Fig. 1. Two transparent sticky traps mounted at a height of 1.45 m and orientated parallel to vines were placed on the north and south side of the middle row of each buckwheat and control plot, 3.7 m apart. An additional four transparent sticky traps were placed on the 1, 3, 6, and 10 rows adjacent to the center of each plot, respectively. Sticky traps were placed in each cardinal direction totaling 10 traps per plot. None of the plots were irrigated over the course of the study.

Individual traps were collected and placed between two clear acetate sheets (21.5 cm × 28 cm, C-line Products, Inc. Mount Prospect, IL) which were labeled with the date the trap was deployed, treatment (buckwheat or control plot), replicate, trap direction (north or south) and side of the trap was facing (open side or foliage side). Traps were stored in a −4 °C freezer until arthropods were counted. Sticky traps were viewed under a dissecting microscope and each arthropod was identified to either family, genus, or species level. The number of marked natural enemies were recorded for each side of each trap to provide data on whether natural enemies were flying towards or away from the buckwheat cover crop or grape canopy.

2.4. Detection of yellow dye

All natural enemies trapped on sticky cards were examined for the presence of the fluorescent yellow dye under UV light in a darkened room. Any group of natural enemy present that contained a mark was included in this study. Yellow dye was detected on leafhopper parasitoids (*A. erythroneurae*), spiders (Araneae), minute pirate bugs (Anthocoridae) and predatory thrips (Aeolothripidae). Unmarked groups of natural enemies that were present on sticky traps were ladybugs (Coccinellidae), lacewings (Chrysopidae), big eyed bugs (Geocoridae), predatory mites (Phytoseiidae and Cunaxidae), and predatory beetles (Carabidae, Scarabaeidae, Anthicidae, and Staphylinidae). Two Croplands SARDI UV flashlights (SARDI, Urrbrae, SA, Australia) were attached to each side of a dissecting microscope with the UV lights illuminating sticky traps. Arthropods with yellow dye directly on their body were considered marked (termed 'contact marked'). Arthropods with yellow dye immediately adjacent to them in the tanglefoot (average distance of yellow dye to natural enemy was 0.66 ± 0.14 mm) on the sticky trap were also considered marked (termed 'allocated marked'). In this instance, it was assumed that yellow dye adjacent arthropod bodies was dislodged as marked arthropods struggled in the tanglefoot (see Corbett and Rosenheim, 1996 for more details on this method). Yellow-marked arthropods were circled with a yellow ultrafine-point indelible marker. Once the entire side of a trap was examined, the top acetate sheet was peeled back, and yellow-marked beneficial arthropods were removed with a toothpick. The toothpick tip containing the marked natural enemy was snapped off and inserted into a 200 μl microcentrifuge tube (Eppendorf North America, Hauppauge, NY), sealed, and labeled. Microcentrifuge tubes were stored at −20 °C until subjected to ELISA testing to detect for the

presence of the albumin and casein protein marks.

2.5. Detection of albumin and casein

Overall, 3,153 natural enemies (∼8% of those captured arthropods), were assayed for the presence of the albumin and casein protein marks. A total of 314 yellow-marked leafhopper parasitoids, *A. erythroneurae,* out of a total of 39,141 parasitic and predatory wasps collected over the course of the study, were removed from sticky traps and examined for the presence of the protein marks. The leafhopper parasitoid, *A. erythroneurae,* accounted for approximately 96% of beneficial Hymenoptera counted on sticky traps (Irvin et al., 2016). All yellow-marked and unmarked spiders (4 yellow-marked/total of 77 [73 unmarked]), minute pirate bugs (3/70), and predatory thrips (5/343) were also removed and examined with ELISA for the presence of protein marks. It was necessary to test arthropods that scored negative for yellow-marks in case these arthropods were protein marked. Therefore, up to 8 leafhopper parasitoids per trap were randomly selected and tested, for a total of 2349 parasitoids, which scored negative for the presence of the yellow dye mark were randomly removed and examined for the presence of egg albumin and casein protein. Each individual arthropod was transferred from its 200 μl microcentrifuge tube to a 2.0 ml microcentrifuge tube containing 1.0 ml of tris buffered saline (TBS) (pH 7.4). Each sample was soaked at 27 °C for a minimum of 1 h at 100 rpm on an orbital shaker. Then, a single 4.5 mm BB (Daisy® Outdoor Products, Rogers, Arkansas, USA) was placed in each microtube and the arthropod sample was thoroughly crushed at 30 Hz for 1 min using a Qiagen TissueLyser (Qiagen Inc., Valencia, CA). Each arthropod sample (100 μl per sample) was assayed for the presence of both marks by the anti-casein and anti-egg albumin ELISAs (Irvin et al., 2012).

The ELISA protocol required eight negative control arthropods per ELISA plate. Therefore, 272 parasitoids, 8 spiders, 8 min pirate bugs, and 24 predatory thrips were removed from sticky traps deployed on July 15, 2008 (before spraying of the triple mark) and used as negative controls. Field-collected arthropods were scored positive for the presence of the respective markers if the ELISA optical density reading exceeded the mean negative control reading by three standard deviations (Hagler, 1997).

2.6. Statistical analyses

Marked natural enemy data were binary and contained many zeros which necessitated pooling capture data across sampling dates. All statistical analyses were performed in SAS (2008) at the 0.05 level of significance. Logistic regression was used to determine the effect of treatment, row side, presence of buckwheat, side of the trap, treatment * row interaction and treatment * side of trap interaction on the percentage of parasitoids marked with yellow dye (allocated marked + contact marked data), albumin, casein and a double mark (where any two of the three marks were detected). Non-significant terms were removed from the model and data were reanalyzed to determine if remaining variables were significant. Pair-wise contrast tests were used to separate means. Where a significant interaction existed, data were analyzed by individual terms. Distance was not included in the logistic regression model since there were no marked parasitoids for some distances or combinations of distances at each treatment, which resulted in no maximum likelihood estimates. Chi-square tests were used to determine the effect of distance at each treatment and the effect of treatment at each distance on the percentage of parasitoids marked with yellow dye (allocated marked + contact marked data), albumin, casein, and a double mark. Fisher's Exact tests were used to separate means.

Data for the percentage of marked spiders, minute pirate bugs, and predatory thrips were pooled together and classified as 'other marked beneficials'. Logistic regression was used to determine the effect of

treatment, row side, presence of buckwheat, side of the trap, treatment * row interaction, treatment * trap side interaction, and species of natural enemy on the percentage of other beneficials marked with yellow dye (allocated marked + contact marked), albumin, casein, and a double mark in the same manner as described for parasitoids. There was no significant (p < 0.05) effect of species of natural enemy on the percentage of beneficial arthropod marked by yellow dye (allocated marked + contact marked), albumin, casein, and a double mark. As such, these data were pooled over species. Chi-square tests were used to determine distance effects in the same manner as described for parasitoids.

The contact marked parasitoid data were analyzed again separately to determine whether those parasitoids that had yellow dye directly on their bodies were captured in control plots. Contact marked parasitoids contained yellow dye directly on their bodies and were a true mark, whereas, allocated yellow dye marks may have contained insects marked by SARDI dust drifting onto the sticky trap, or insects marked by protein obtained by an insect walking over dried protein. Fisher's exact test was used to determine whether treatment and distance of capture were independent (McDonald, 2009). Separate chi-square tests were used to determine the effect of treatment (pooling data over distance) and distance (pooling data over treatment) on the number of parasitoids with yellow dye directly on their bodies.

3. Results

3.1. Comparing yellow dye (allocated + contact marked), albumin and casein marked arthropods

No triple marked arthropods were captured during the course of this study. Arthropods collected on sticky traps were either unmarked, had a single mark (yellow dye, albumin protein, or casein protein) or had a double mark. For parasitoids marked with yellow dye, albumin, casein, or a double mark and 'other beneficials' marked with yellow dye, albumin, or a double mark, a significantly higher percentage were captured on traps placed amongst flowering buckwheat plants treated with the marking solution compared with traps deployed in the absence of buckwheat plants (Tables 1 and 2; Fig. 2). There was no significant effect of buckwheat presence on the percentage of 'other beneficials' marked with casein (Table 2; Fig. 2).

The percentage of parasitoids marked with albumin was significantly higher (i.e., more than 2-fold) on the foliage side of the trap, compared with the open side (Fig. 3a; Table 1). In contrast, the percentage of parasitoids marked with a double-mark was more than 2-fold higher on the open side of the trap, compared with the foliage side (Fig. 3a; Table 1). There was no significant effect of trap side on the percentage of parasitoids marked with yellow dye or 'other beneficials' marked by yellow dye, albumin, casein, and a double-mark (Tables 1 and 2). For parasitoids marked with casein there was a significant treatment * trap side interaction effect (Table 1). In buckwheat plots, the traps facing the grape foliage captured 1.4-fold more parasitoids marked with casein compared with the open side (Fig. 3b). In control

plots, the traps facing into the open row captured 1.4-fold more parasitoids marked with casein compared with the foliage side (Fig. 3b).

A total of 339 parasitoids were marked with yellow dye. The maximum percentage (45%) of parasitoids marked with yellow dye occurred in the middle of buckwheat plots (Fig. 4a). There was a significantly higher percentage of parasitoids marked by yellow dye in buckwheat plots at distances 0, 1, 6, and 10 rows from the middle of the buckwheat plot compared with control plots at the same distances (Fig. 4a; Table 3). The percentage of parasitoids marked with yellow dye captured 10 rows from the middle of buckwheat plots was 3-fold higher in buckwheat plots compared with control plots suggesting that parasitoids marked with yellow dye dispersed at least 10 rows adjacent to buckwheat refuges (Fig. 4a; Table 3).

The maximum percentage (5%) of parasitoids marked with albumin occurred in the middle of buckwheat plots (Fig. 4b). Parasitoids marked with albumin were also captured three rows from buckwheat plots, and this was significantly higher compared with marked parasitoids captured in control plots at the same distance (Fig. 4b; Table 3). This indicated that parasitoids marked with albumin dispersed three rows adjacent to buckwheat refuges. No parasitoids marked with albumin were captured 6 rows from buckwheat plots, whereas, 0.8% of parasitoids captured 6 rows from the middle of control plots were marked by albumin (Fig. 4b; Table 3).

The maximum percentage (3%) of parasitoids marked with casein occurred 10 rows from the middle of buckwheat plots. However, at this distance there was no significant difference in the percentage of parasitoids marked with casein between buckwheat and controls plots (Fig. 4c; Table 3). A significantly higher (approximately 2–3 times higher) percentage of parasitoids marked with casein were captured in buckwheat plots compared with control plots at distances 0, 1, and 6 rows from the middle of these plots (Fig. 4c; Table 3). No parasitoids marked with casein were captured 3 rows from buckwheat plots, whereas, 3% of parasitoids captured 3 rows from the middle of control plots were marked by casein (Fig. 4c; Table 3).

The maximum percentage (6%) of parasitoids marked with a double mark where any two out of the three marks tested positive occurred in the middle of buckwheat plots (Fig. 4d). For control plots, no parasitoids with a double mark were captured for any of the five dispersal distances measured. The percentage of parasitoids with a double mark in buckwheat plots ranged from 0.3 to 1% at distances 1, 3 and 10. However, these differences were not significantly different to the zero doubled marked parasitoids captured in control plots for the same distances (Fig. 4d; Table 3).

A total of 3 (out of a total of 73), 4 (total of 77) and 5 (total 343) minute pirate bugs, spiders and predatory thrips, respectively, were marked with yellow dye (contact + allocated marks). The maximum percentage (17–22%) of these 'other beneficials' marked with yellow dye, albumin or a double-mark occurred in the middle of buckwheat plots, and for albumin and double-marked parasitoids, this was significantly higher than the zero captures in the middle of the control plots (Fig. 5a, b and d; Table 3). 'Other beneficials' marked with yellow dye, albumin, or double-mark were also captured 1 and 3 rows from the

Table 1

Chi-square and p-values for a logistic regression model analyzing the effect of treatment, row side (north versus south), buckwheat presence, trap side (open versus foliage), treatment * row interaction and treatment * trap side interaction on the number of leafhopper parasitoids, *Anagrus erythroneurae* (Hymenoptera: Mymaridae), marked by yellow dye, casein, albumin or two of these three marks (df = 1 for all tests; n/a = maximum likelihood estimate for these factors do not exist since there were no marked parasitoids on some levels of these factors for use in analyses).

Insect group and mark	Treatment		Row side		Buckwheat presence		Side of trap		Treatment * Row side		Treatment * side	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Parasitoids marked by yellow dye	64.58	< 0.0001	4.80	0.03	293.58	< 0.0001	0.01	0.92	10.35	0.001	1.03	0.30
Parasitoids marked by albumin	9.15	0.003	7.78	0.005	86.88	< 0.0001	57.08	< 0.0001	9.186	0.002	2.03	0.15
Parasitoids marked by casein	61.24	< 0.0001	7.01	0.01	69.73	< 0.0001	13.29	0.0003	5.38	0.02	29.24	< 0.0001
Parasitoids with double-mark	n/a	n/a	18.23	< 0.0001	124.52	< 0.0001	13.84	0.0002	n/a	n/a	n/a	n/a

Table 2

Chi-square and p-values for a logistic regression model analyzing the effect of treatment, row side (north versus south), buckwheat presence, trap side (open versus foliage), treatment * row interaction and treatment * trap side interaction on the number of 'other beneficial arthropods' (i.e., spiders, predatory thrips, and minute pirate bugs) marked by yellow dye, casein, albumin or two of these three marks (df = 1 for all tests except for species where df = 2; n/a = maximum likelihood estimate for these factors do not exist since there were no marked 'other beneficials' on some levels of these factors for use in analyses).

Insect group and mark	Treatment		Row side		Buckwheat presence		Side of trap		Species		Treatment * Row side		Treatment * side	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Other beneficials marked by yellow dye	0.01	0.92	0.89	0.34	18.42	< 0.0001	0.01	0.91	4.71	0.09	2.48	0.11	0.02	0.91
Other beneficials marked by albumin	0.068	0.79	0.64	0.42	28.60	< 0.0001	2.06	0.15	0.08	0.96	0.18	0.67	n/a	n/a
Other beneficials marked by casein	0.02	0.83	0.96	0.32	0.80	0.37	0.48	0.48	3.31	0.19	0.42	0.51	3.99	0.04
Other beneficials with double-mark	0.69	0.40	2.18	0.13	23.24	< 0.0001	0.91	0.33	0.34	0.84	n/a	n/a	n/a	n/a

middle of buckwheat plots. However, these levels of marked natural enemies were not significantly different than those captured in control plots at the same distances (1 and 3) (Fig. 5a, b and d; Table 3).

3.2. Comparing yellow dye (contact marked) arthropods

Of a total of 339 marked parasitoids, 10% contained yellow dye directly on their bodies (contact mark) and 90% of marked parasitoids were allocated as being marked because yellow dye was within 0.66 ± 0.14 mm of their bodies (allocated mark). For the contact marked parasitoids, treatment and distance were independent variables (Fisher Exact test, p = 0.15). There were five times more contact marked parasitoids captured in buckwheat plots (26 parasitoids captured) compared with control plots (5 parasitoids) (X^2 = 15.13, df = 1, p < 0.001). There was no significant effect of dispersal distance from the center of the buckwheat plots on the number of marked parasitoids containing yellow dye directly on their bodies (X^2 = 6.44, df = 4, p = 0.17).

Of marked spiders, 50% contained a yellow contact mark (2 contact marked / 2 allocated marked), and 50% of contact marked spiders (i.e., one spider) were captured in buckwheat plots. Of marked pirate bugs, 67% contained a contact mark (2 contact marked / 1 allocated mark) and 100% of contact marked pirate bugs were captured in buckwheat plots. All five predatory thrips marked with yellow dye were assigned as allocated marks and captured in buckwheat plots.

4. Discussion

Up to 28 times more parasitoids marked by yellow dye, albumin,

and casein, and up to 40 times more 'other beneficials' marked by yellow dye and albumin were captured on sticky traps placed near buckwheat plants sprayed with the triple marking solution compared to control plots lacking buckwheat. This indicates that the triple mark was successful at marking minute parasitoids and other beneficial arthropods in the field with at least one mark. Laboratory studies showed that yellow dye and casein were the most efficient marking methods in topical applications the laboratory, detecting up to 29% more marked mymarids compared with albumin (Irvin et al., 2012). In contrast, results from the current study suggested that yellow dye and albumin may be the most reliable methods for marking minute Hymenoptera in the field. Casein resulted in a higher ratio of marked mymarids in control versus buckwheat plots when compared with yellow dye and albumin. This may suggest that casein resulted in a higher degree of false positives in control plots compared with yellow dye and albumin. The less consistent results of casein compared with albumin may be attributable to differences in ability of beneficial arthropods to "self-mark". Irvin et al. (2012) demonstrated in the laboratory that mymarids were able to self-mark by walking over dried casein residues, whereas, mymarids were unable to self-mark when exposed to dried albumin residues. In the current field study, wind may have carried dried flakes of the marking spray into control plots allowing beneficial arthropods to self-mark with casein.

Previous studies have demonstrated the use of albumin and casein to successfully mark pest flies, thrips, generalist predators, and parasitoids in the field (Horton et al., 2009, Hagler et al., 2014, Klick et al., 2014, Swezey et al., 2014, Fernandes and Fernandes, 2015). The use of albumin and casein for marking parasitoids is relatively inexpensive and can be quickly and consistently applied over large areas using

□ Buckwheat absent   ■ Buckwheat present
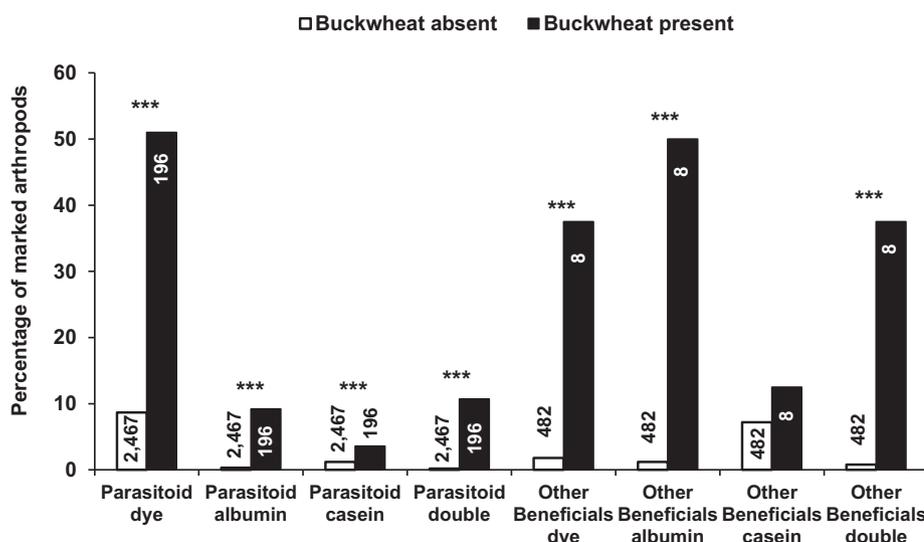
Fig. 2. Effect of presence or absence of a flowering buckwheat cover crop treated with a triple-marking solution on the percentage of marked leafhopper parasitoids, *Anagrus erythroneurae* and 'other beneficial arthropods' (i.e., minute pirate bugs, spiders, and predatory thrips) captured on transparent sticky traps located in a vineyard in southern California (numbers in columns indicate sample size; asterisk indicates a significant difference [p < 0.001] between traps placed within flowering buckwheat treated with a triple marking solution or in absence of flowering buckwheat; double = a double mark in which any two of yellow dye, albumin, or casein tested positive).
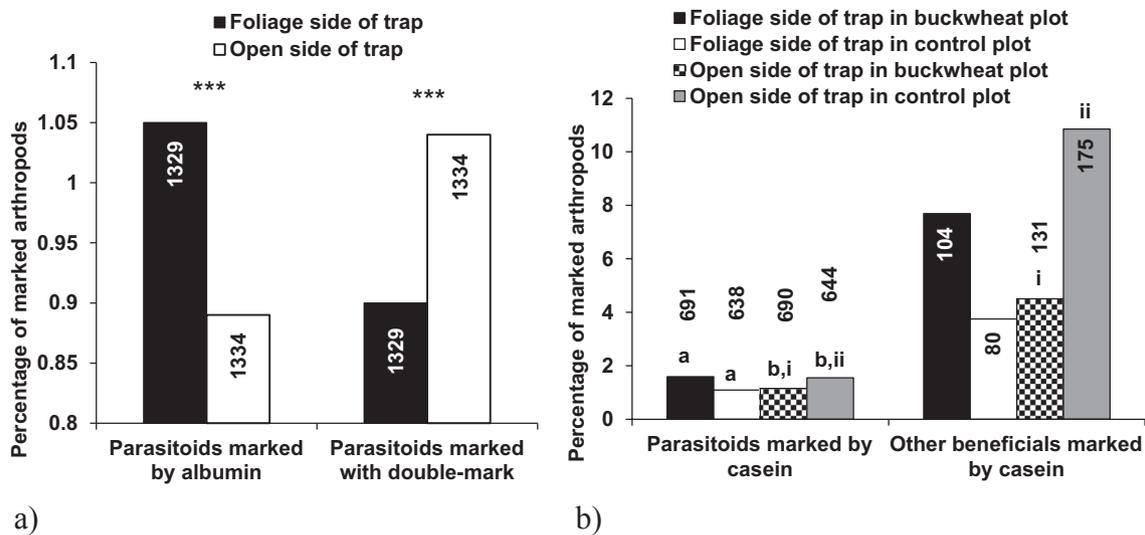
Fig. 3. Effect of treatment and trap side (open versus foliage side) on the percentage of marked 'other beneficial arthropods' (i.e., minute pirate bugs, spiders and predatory thrips) captured on transparent sticky traps in buckwheat and control plots located in a vineyard in southern California (numbers in columns indicate sample size; asterisks indicate significant [$p < 0.001$] differences between trap sides within buckwheat and control plots combined; different letters indicate significant [$p < 0.05$] differences between trap sides within each treatment plot separately; different Roman numerals indicate significant [$p < 0.05$] differences between treatments for each trap side; double mark = when any two of yellow dye, albumin, or casein tested positive).
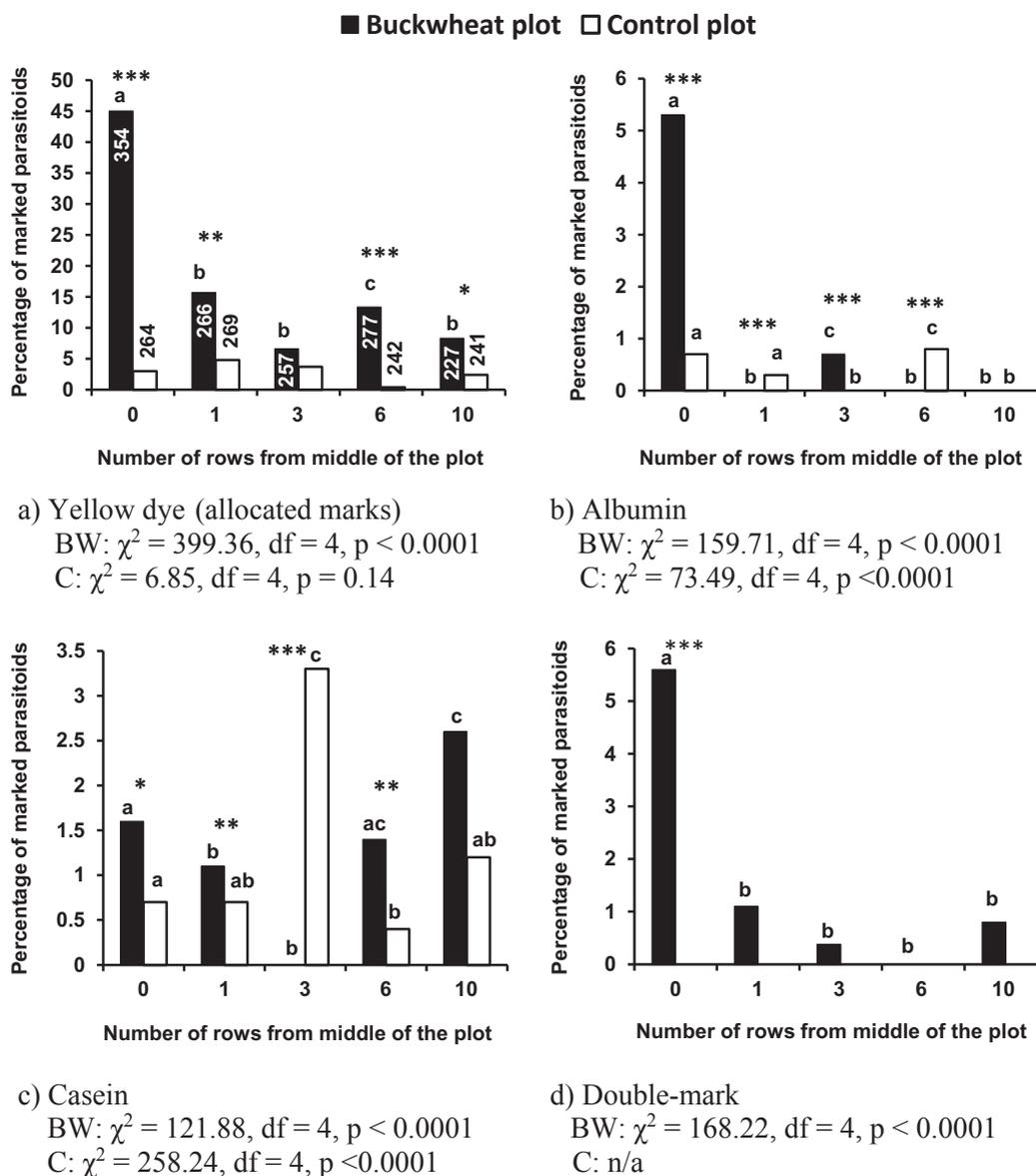
commercial spray equipment (Horton et al., 2009).

Results comparing the percentage of 'other beneficials' marked by yellow dye (allocated + contact marked) between distances in buckwheat plots indicated that marked spiders, predatory thrips, and minute pirate bugs dispersed into the third row (i.e., 9 m) adjacent to buckwheat refuges. In California vineyards, mymarid egg parasitoids are of particular importance for controlling key pests of grape. Three species of leafhoppers are a major economic concern and are controlled to varying degrees by some combination of three *Anagrus* species (Wilson and Triapitsyn, 2017). Similarly, three *Gonatocerus* species were released in California to control *H. vitripennis* (CDFA, 2003). Comparing the percentage of parasitoids marked by yellow dye (allocated + contact marked) between control and buckwheat plots demonstrated that there was a significantly higher percentage of marked mymarids captured up to six (i.e., 18 m) and ten rows (i.e., 30 m) from the middle of plots. This strongly suggests that mymarids can disperse at least 30 m into vineyards from the center of cover crop refuges. This finding is in accordance with other marking studies have shown that mymarids can disperse 24.5 m per day (Corbett and Rosenheim, 1996), while other parasitoids can disperse from cover crops at a rate of 16–20 m per day (Langhof et al., 2005, Lavandero et al., 2005, Wanner et al., 2007), or in some instances, up to 30 m (Freeman-Long et al., 1998).

Approximately 0.25% of mymarids captured at all distances from control plots were marked with yellow dye. This may indicate the baseline for falsely marked parasitoids which could result in an over-estimate of numbers of insects marked and distances dispersed. Up to 17% of mymarids and other beneficials marked with yellow dye, albumin, or casein were captured in non-sprayed control plots. This finding may indicate an assay error or that wind either carried dried flakes or caused the marking spray containing yellow dye, albumin and casein to drift into control plots where it marked arthropods. However, the likelihood and significance of dried marking residues being move by the wind into untreated plots or onto sticky traps used to collect arthropods during marking studies is unknown. Alternatively, it is possible that some arthropods were able to disperse the 36 m between

buffer zones from sprayed buckwheat plots into the non-sprayed control plots. Five mymarids and two spiders that contained yellow dye directly on their bodies (contact marked) were captured on sticky traps in control plots which supports the possibility that *A. erythroneurae* and some spider species were able to disperse further than the 36 m buffer zones separating buckwheat and control plots. This finding illustrates the importance of using large buffer zones between treatments in marking trials investigating the arthropod dispersal.

Laboratory studies investigating the use of the triple mark containing yellow dye, albumin, and casein for marking mymarids demonstrated that relying on a double mark where any two of the three marks test positive may be more reliable and result in a lower percentage of false positives compared to relying on one mark (Irvin et al., 2012). In the current field study, results comparing the percentage of parasitoids marked with a double mark between distances in buckwheat plots indicated that double marked mymarids were found up to 10 rows from buckwheat refuges, while no double marked mymarids were captured in control plots. This finding suggests that mymarids dispersed at least 30 m from buckwheat refuges in 6 days. Results comparing the percentage of 'other beneficials' marked with a double mark across distances 0–30 m from buckwheat plots indicated that some natural enemies can disperse at least three rows (i.e., 9 m) from buckwheat refuges within 6 days.

Under laboratory conditions (26 ± 2 °C, 30–40% R. H., 16L:8D photoperiod with fluorescent lighting), the triple mark used in field experiments (i.e., yellow dye, albumin, and casein) was retained on marked mymarids for at least 11 days (Irvin et al., 2012). The current field study was conducted under hot, dry summer conditions with no rainfall. Average air temperature was 23.8 ± 0.6 °C (maximum recorded air temperature was 32.8 °C), relative humidity 18–85%, photoperiod 14L:10D, and average wind speed was 1.9 m/s (CIMIS weather database; Station 44; http://wwwcimis.water.ca.gov/Default.aspx). It is unknown how high maximum day time temperature and solar radiation affects the persistence of the triple mark in the field when compared to laboratory estimates. It is likely that the triple mark was
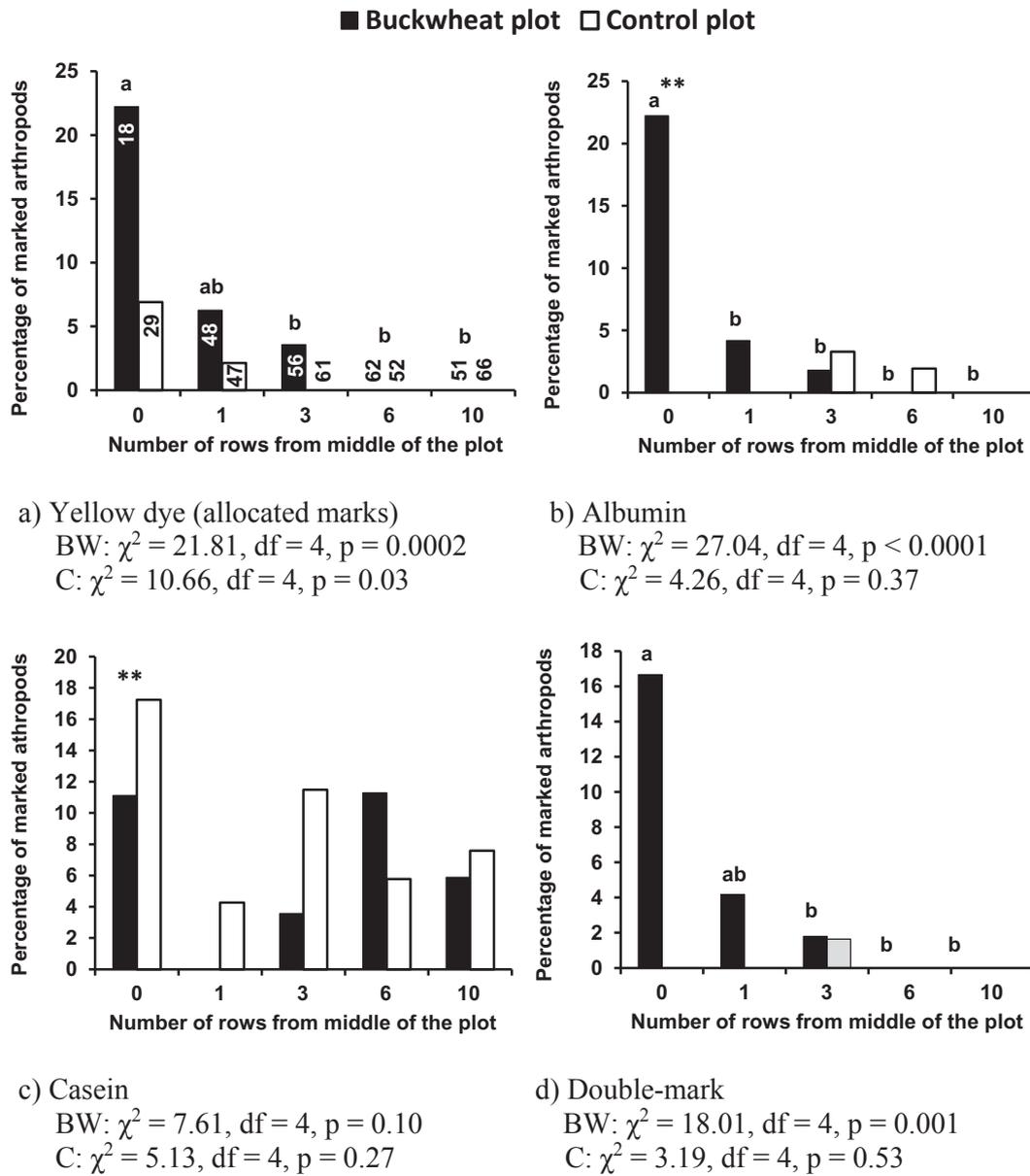
■ Buckwheat plot □ Control plot

a) Yellow dye (allocated marks)
   BW: $\chi^2 = 399.36$, df = 4, p < 0.0001
   C: $\chi^2 = 6.85$, df = 4, p = 0.14

b) Albumin
   BW: $\chi^2 = 159.71$, df = 4, p < 0.0001
   C: $\chi^2 = 73.49$, df = 4, p <0.0001

c) Casein
   BW: $\chi^2 = 121.88$, df = 4, p < 0.0001
   C: $\chi^2 = 258.24$, df = 4, p <0.0001

d) Double-mark
   BW: $\chi^2 = 168.22$, df = 4, p < 0.0001
   C: n/a

Fig. 4. Percentage of leafhopper parasitoids, *Anagrus erythroneurae* marked by a) yellow dye, b) albumin, c) casein, or d) a double-mark (in which any two of yellow dye, albumin or casein tested positive) captured on sticky traps deployed at five distances from the middle of buckwheat plots sprayed with a solution containing yellow dye, albumin, and casein and no spray control plots (numbers in columns of Fig. 4a indicate sample size which are equivalent to samples sizes for Fig. 4b–d; asterisks indicate significant [* = p < 0.05, ** = p < 0.01, *** = p < 0.001] differences between buckwheat and control plots; Chi square test statistics show overall effect of distance at each treatment; BW = buckwheat; C = control; n/a = not applicable since no marked insects were captured across all distances).

Table 3
Effect of treatment at each distance for Chi-square test on percentage of marked leafhopper parasitoids *Anagrus erythroneurae* (Hymenoptera: Mymaridae) and 'other beneficial arthropods' (i.e., spiders, predatory thrips, and minute pirate bugs) (df = 1 for all tests).

Insect group and mark	Distance (number of rows) from middle of buckwheat or control plots									
	0		1		3		6		10	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Parasitoids marked by yellow dye	145.29	< 0.0001	8.46	0.004	2.78	0.09	37.93	0.0001	4.17	0.04
Parasitoids marked by albumin	12.99	0.0003	26.51	< 0.0001	26.05	< 0.0001	33.02	< 0.0001	n/a	n/a
Parasitoids marked by casein	4.88	0.02	8.37	0.004	132.74	< 0.0001	8.59	0.003	0.68	0.41
Parasitoids with double-mark	38.96	< 0.0001	2.27	0.13	1.13	0.29	n/a	n/a	1.59	0.21
Other beneficials marked by yellow dye	2.34	0.13	1.00	0.32	2.21	0.14	n/a	n/a	n/a	n/a
Other beneficials marked by albumin	7.04	0.008	2.00	0.16	0.26	0.60	1.20	0.27	n/a	n/a
Other beneficials marked by casein	0.32	0.56	2.08	0.14	2.56	0.10	1.07	0.29	0.12	0.71
Other beneficials with double-mark	5.16	0.02	2.00	0.16	0.003	0.95	n/a	n/a	n/a	n/a

n/a = not applicable since zero marked insects were captured for both treatments.

■ Buckwheat plot □ Control plot

a) Yellow dye (allocated marks)
BW: $\chi^2 = 21.81$, df = 4, p = 0.0002
C: $\chi^2 = 10.66$, df = 4, p = 0.03

b) Albumin
BW: $\chi^2 = 27.04$, df = 4, p < 0.0001
C: $\chi^2 = 4.26$, df = 4, p = 0.37

c) Casein
BW: $\chi^2 = 7.61$, df = 4, p = 0.10
C: $\chi^2 = 5.13$, df = 4, p = 0.27

d) Double-mark
BW: $\chi^2 = 18.01$, df = 4, p = 0.001
C: $\chi^2 = 3.19$, df = 4, p = 0.53

Fig. 5. Percentage of 'other beneficial arthropods' (i.e., minute pirate bugs, spiders, and predatory thrips) marked by a) yellow dye, b) albumin, c) casein or d) a double-mark (in which any two of yellow dye, albumin or casein tested positive) captured on sticky traps deployed at five distances from the middle of buckwheat plots sprayed with a solution containing yellow dye, albumin and casein and no spray control plots (numbers in columns of Fig. 5a indicate sample size which are equivalent to samples sizes for Fig. 5b–d; asterisks indicate significant [p < 0.01] differences between buckwheat and control plots; Chi square test statistics show overall effect of distance at each treatment; BW = buckwheat; C = control).

retained on marked mymarids and sprayed foliage for the duration of the experiment since there was no precipitation during the course of the study. Precipitation is a significant factor interfering with protein mark acquisition and retention (Jones et al., 2006, Klick et al., 2014, Hagler et al., 2014).

To exploit the dispersal capabilities of natural enemies, results presented here suggest that buckwheat refuges planted in California vineyards could be planted every 6th (i.e., 18 m) or 10th (30 m) row to provide natural enemies access to flowering buckwheat refuges. Despite the potential of cover crops for enhancing natural enemy activity in southern California vineyards, results by Irvin et al (2016) suggest that

the negative effects of an irrigated buckwheat cover crop may exceed benefits. The downsides for using cover crops in arid grape production areas in southern California include competition with the crop, increased risk of insect pest and disease prevalence, lower crop quality (e.g., reduced brix levels in grapes), and increased landscape management costs including the extra expense associated with sowing, irrigating, and maintaining the cover crop (Irvin et al., 2016). Increased humidity and higher frost risk may also result from cover crop plantings (Miller et al., 1989). Consequently, when planning and implementing conservation biological control programs, all aspects of the potential cover crop under consideration, positive and negative, need to be

evaluated when deciding if their use for pest management is warranted.

Acknowledgements

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