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Laboratory investigation of triple marking the parasitoid *Gonatocerus ashmeadi* with a fluorescent dye and two animal proteins

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Abstract

Gonatocerus ashmeadi Girault (Hymenoptera: Mymaridae), a parasitoid of *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) (glassy-winged sharpshooter), was used as a model insect to investigate triple marking a minute hymenopteran for potential use for monitoring dispersal patterns of natural enemies in the field. The triple mark contained egg albumin in chicken eggs, casein in bovine milk, and SARDI yellow fluorescent dye. Three application treatments of the triple mark were investigated: (1) a wet topical treatment, (2) a dry residue treatment, and (3) untreated control. The presence of albumin and casein protein marks were detected by an anti-albumin and anti-casein enzyme-linked immunosorbent assays (ELISA) using both 'soaked' and 'crushed' specimens. Of the topically treated parasitoids, yellow dye, casein, and albumin were detected on 88, 69, and 21% of the crushed samples, respectively. The yellow dye and casein (tested with crush ELISA) were the most efficient marking methods, detecting up to 29% more marked *G. ashmeadi*. Yellow dye resulted in zero false positives in the untreated control. The percentage of false positives for casein (tested with crush ELISA) was 1.3; however, this was reduced to 0% when a double-marking system using any two of the three marks (yellow dye, casein, and albumin) were used to mark parasitoids. This double-mark system resulted in 65% of parasitoids being successfully marked in the topical treatment over the duration of the study. For casein, crush ELISA was 26% more sensitive and 24% more accurate than soak ELISA for detecting this mark. Yellow dye, albumin, and casein (tested with crush ELISA) were retained on marked parasitoids for the entire duration of the 11-day study. Parasitoids self-marked with yellow dye, albumin (tested with soak ELISA), casein (tested with crush ELISA), and the double-mark (tested with crush ELISA) by walking over dried residue of the triple mark. This resulted in up to 17% more marked parasitoids in the residue treatment compared with the untreated control. A topical application of the triple mark had no effect on survival of *G. ashmeadi* compared with the control. The residue treatment resulted in significantly lower mortality than the untreated control, indicating that *G. ashmeadi* may have fed on the protein in the residue of the triple-mark, which enhanced longevity.

Introduction

Numerous marking and tracking techniques have been developed to investigate the movement of insects to study their abundance, dispersal, and survival in the field. In biological control, an efficient marker is paramount for investigating the dispersal characteristics of natural enemies (Corbett & Rosenheim, 1996; Weisser, 2000; Canto-Silva et al., 2006; Hougardy & Mills, 2006; Scarratt et al., 2008; Schellhorn et al., 2008; Petit et al., 2009) and predatory–prey/parasitoid–host interactions (Hagler, 2006; Wanner et al., 2006b). The most suitable marker for any given study is strongly influenced by whether a mark-release-recapture or mark–capture study is being

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conducted. A mark–capture type study is the most difficult because insects need to be marked directly in the field and often over large areas. Furthermore, methods available to mark small and delicate parasitoids are lacking (Hagler & Jackson, 2001). Fluorescent powders and dyes have been used to investigate movement and behavior of parasitoids (Schellhorn et al., 2004; Reeve & Cronin, 2010). Although the use of powders are an inexpensive way to mark large populations of insects, these visible markers are not always effective at marking targets and they may give false negatives (Hagler & Miller, 2002). Moreover, powders and dyes are heavy and may negatively affect survival and dispersal behavior of minute Hymenoptera (Messing et al., 1993; Corbett & Rosenheim, 1996). Rare or trace elements such as rubidium chloride are probably the most useful markers for labeling minute Hymenoptera. They are environmentally safe and can be successfully retained in or on many insects (Jackson et al., 1988; Corbett et al., 1996; Wanner et al., 2006a). However, high concentrations of trace elements have been shown to be deleterious to many insects (Stimmann et al., 1973; Van Steenwyk et al., 1978) and the analytical methods used to detect the presence of trace elements are time consuming, require technical expertise, and involve specialized and costly equipment (Akey et al., 1991; Hagler & Jackson, 2001).

Hagler et al. (1992) developed a protein marking technique that overcomes many of the drawbacks listed above. Immunomarking involves marking insects with an exogenous protein and detecting the mark by an anti-protein-specific enzyme-linked immunosorbent assay (ELISA). Vertebrate proteins, such as rabbit and chicken immunoglobulin G (IgG), have been effective for marking a variety of different insects, including parasitoids (Hagler, 1997; Hagler & Jackson, 1998; Hagler et al., 2002; Janke et al., 2009). However, these IgG proteins are too expensive for mark–capture studies, which involve marking insects directly in the field using conventional spray rigs. Recently, this drawback was overcome with the development of a second generation of protein-specific ELISAs. Specifically, these ELISAs are designed to detect egg albumin in chicken egg whites, soy trypsin protein in soy milk, and bovine casein protein in milk. These readily available food products are 350–2 000 times less costly than the highly purified vertebrate IgG proteins (Jones et al., 2006). Moreover, the ELISA tests for these food proteins are simple, inexpensive, sensitive, and have been standardized for mass production (Hagler & Jones, 2010).

This is the first study that investigates the use of inexpensive albumin and casein to mark minute Hymenoptera. In addition, the current study combined these two protein markers with a fluorescent yellow dye to make a novel triple-marking compound. Hagler & Miller (2002) suggested that these proteins could be combined with conventional marking procedures such as visible dusts and dyes to help provide a fail-safe method to ensure that 100% of the insects are marked. A double- or triple-marking system may also eliminate the number of false positives so that no insects are incorrectly marked positive.

Here, we tripled marked *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae), a small (1.28–1.76 mm long; Triapitsyn, 2006) egg parasitoid of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae), with egg albumin, milk casein, and yellow dye. Our goals were to determine: (1) whether *G. ashmeadi* (males and females) could be successfully marked, (2) the mode by which *G. ashmeadi* were marked (e.g., by direct topical spray or residual contact) most effectively, (3) how long parasitoids retained marks, and (4) the lethality of the mark.

Materials and methods

Insect colonies and laboratory conditions

Laboratory colonies of *H. vitripennis* and *G. ashmeadi* were maintained at the University of California, at Riverside (UCR). *Citrus limon* (L.) Burm. F. cv. 'Eureka' trees (Rutaceae), ca. 2 years old and grafted to *Citrus marcophylla* Wester rootstock, were obtained from C & M Nurseries, Nipomo, CA, USA. Trees were pruned to 60 cm in height, potted into 4-l containers, and fertilized every 2 weeks with Miracle-Gro (20 ml/3.5 l of water; Scotts Miracle-Gro Products, Marysville, OH, USA). Oviposition cages (61 × 61 × 61 cm, 'BugDorm 2'; BioQuip Products, Rancho Dominguez, CA, USA) containing two 'Eureka' trees and ca. 200 field-collected *H. vitripennis* were set up in a temperature-controlled greenhouse (26 ± 4 °C, 12–35% r.h., and natural lighting). Each cage was supplemented with ca. 50 field-collected *H. vitripennis*, on 5 days in every week. Trees exposed to *H. vitripennis* were checked for egg masses every 1–3 days and leaves bearing egg masses were excised. Excised leaves with egg masses 1–3 days of age (a preferred age for *G. ashmeadi* oviposition; Irvin & Hoddle, 2005) were transported to the laboratory and placed into Petri dishes (10 × 1.5 cm; Becton Dickinson Labware, Franklin Lakes, NJ, USA) lined with moist filter paper (9 cm; Whatman International, Maidstone, UK). Droplets of 50% honey-water solution (3:1 vol/vol, Natural uncooked honey; Wild Mountain Brand, Oakland, CA, USA) were placed on the lid of each Petri dish. Two mated female *G. ashmeadi* were introduced into each Petri dish for oviposition and removed after 3 days. Filter paper was moistened with water every 2–3 days as needed. Petri dishes were held at 26 ± 2 °C and 30–40% r.h. under a L16:8D photoperiod and checked daily for

parasitoid emergence. Newly emerged (<12 h) female and male *G. ashmeadi* were aspirated into clean 130-ml plastic vials (40 dram Plastic Vial; Thornton Plastics, Salt Lake City, UT, USA) and 50% honey-water was supplied in droplets on the lid before use in marking experiments.

Experimental design

The spray used to mark parasitoids contained three marks consisting of 20% chicken egg white (strained All Whites; Papetti Foods, Elizabeth, NJ, USA) (containing ca. 5% albumin; Anonymous 1, 2011), 78% milk (Ralphs 2% Reduced Fat Milk; Inter-American Products, Cincinnati, OH, USA) (containing ca. 80% casein; Anonymous 2, 2011), and 2% yellow SARDI fluorescent pigment (liquid dye) (Topline Paint, Aldelaide, SA, Australia). The recommended rate for application of the yellow fluorescent dye as an agricultural spray is between 1 and 2 l per 100 l (i.e., 1–2%) of agricultural spray (e.g., insecticide or herbicide applications). Between 22 July and 19 August 2007, seven replicates of three spray treatments were set up in a randomized block design. The spray treatments included: (1) 'wet topical treatment': mark was sprayed directly onto parasitoids inside the cage and parasitoids remained in the cage after the spray dried; (2) 'dry residue treatment': mark was sprayed inside the cage and parasitoids were introduced 2 h after the mark dried; and (3) an untreated control.

Each replicate consisted of a wooden cage (32 × 34 × 37 cm) painted white, with a glass top, white mesh back for ventilation, and hinged front door containing a canvas sleeve for access. Cages were held in the laboratory at 26 ± 2 °C and 30–40% r.h. under a L16:8D photoperiod with fluorescent lighting. One vial containing 45–90 newly emerged male and female parasitoids (the number of parasitoids treated was dependent on daily emergence, and parasitoid sex ratio averaged 19% males) was placed into the center of each cage. Parasitoids were released into treatment cages by removing the lid of the vial and gently tapping the vial on the bottom of the cage to dislodge parasitoids. For the wet topical and dry residue treatments, the spray was applied into each cage through the cloth sleeve in the door using eight pumps (total volume of spray per cage = 5.83 ± 0.03 ml) of a 900-ml fine-mist hand sprayer (The Home Depot All Purpose Sprayer; Enviro-Kind, Atlanta, GA, USA) operated inside the cage. The direction of the spray was changed for each pump to increase spray coverage throughout the cage. For the dry residue treatment, the spray was left to dry for 2 h before parasitoids were released into cages. In addition, water and honey-water was provided to parasitoids 2 h after set up in all treatment replicates. Water was supplied via a 7.4-ml glass vial (2 dram Fisherbrand Glass Vial; Fisher Scientific, Pittsburgh, PA, USA) with a 5-cm cotton wick, which was placed on the bottom of each cage and replenished daily using a separate 236 ml wash bottle (Nalagene; Thermo Fisher Scientific, Rochester, NY, USA) dedicated to each treatment to prevent cross contamination. Honey-water was supplied to parasitoids by adhering two 2.5-cm strips of yellow-colored paper with droplets of 50% honey-water to the top of each cage. Paper strips with honey were replaced daily. Parasitoid mortality was recorded daily and dead parasitoids were removed. Every other day, for 1–11 days after spray application, five live *G. ashmeadi* were removed from each cage by placing a clean 130-ml plastic vial over a randomly selected parasitoid located inside the cage surface and slipping the lid under the vial. Vials containing parasitoids were labeled with a unique replicate number (correlating with the treatment, replicate, and day the parasitoid was removed) and immediately placed in a freezer at −20 °C.

Yellow dye inspections

Parasitoids were killed by freezing, sexed, and viewed under UV light for presence of yellow fluorescent dye. This was conducted by placing the parasitoid on a clean white 130 plastic vial lid under a dissecting microscope in a dark room. Two Croplands SARDI UV flashlights (SARDI, Urrbrae, SA, Australia) were held on either side of the dissecting microscope with the UV lights illuminating the parasitoids. Each parasitoid was given a score out of five representing the amount of body coverage with yellow dye. A score of 0 indicated no presence of the yellow dye; one speck of yellow dye was given a score of 1; two specks or up to 10% coverage equaled a score of 2; 11–20% coverage was given a score of 3; 21–40% coverage equaled a score of 4, while a score of 5 indicated above 40% coverage. Parasitoids with a score of 1 or more were recorded as testing positive for the yellow dye mark. During this process, it was observed that parasitoids had fed on the triple mark because a yellow glow was seen inside the abdomen. This was also recorded as a positive result for the yellow dye. After examination for yellow dye marks, parasitoids were placed in individual 2.0-ml microcentrifuge tubes (Eppendorf Safe Lock Tubes; Eppendorf, Hamburg, Germany), labeled with parasitoid replicate and frozen at −20 °C. Parasitoids were shipped on ice overnight from UCR to the USDA-ARS Arid-Land Agricultural Research Center in Maricopa, AZ, USA for detection of albumin and casein protein following the ELISA protocols described below. *Gonatocerus ashmeadi* serving as negative controls were fed honey-water for 24–48 h after emergence. Negative controls were required as part of the ELISA methods outlined below.

ELISA testing

Each individual parasitoid was examined for the presence of casein and egg albumin protein after soaking or crushing the sample. First, each 2.0-ml microcentrifuge tube containing a single parasitoid was filled with 1 000 µl of tris-buffered saline (TBS) (pH 7.4) and soaked at 27 °C for a minimum of 1 h at 100 r.p.m. on an orbital shaker. The soaked sample served to test the parasitoid for the presence of an external protein mark. A 100-µl aliquot of each soaked sample was added to a well of an ELISA plate and assayed for the presence of casein protein, and a second 100 µl sample was added to another ELISA plate and assayed for the presence of egg albumin protein (Jones et al., 2006). Then, a single 4.5-mm BB (Daisy® Outdoor Products, Rogers, AR, USA) was placed in each microtube and the remaining 800 µl insect sample was thoroughly crushed at 30 Hz for 1 min using a Qiagen TissueLyser (Qiagen, Valencia, CA, USA). The crushed sample served to test the parasitoid for proteins that it may have ingested (e.g., an internal mark). Again, a 100-µl aliquot was taken from each sample and assayed for the presence of both marks.

Unmarked *G. ashmeadi* serving as negative controls were fed honey water for 24–48 h after emergence and assayed by each ELISA (n = 8 per ELISA plate). Individual parasitoid samples collected from each cage 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).

Statistical analysis

All statistical analyses were conducted in SAS Institute (1990). The following Logistic Regression Model (1) was used to determine the effect of treatment (wet topical treatment, dry residue treatment, and an untreated control), day (days after marking; 1–11 days) and parasitoid sex for each marking method (albumin, casein, and yellow dye):

$$\text{Logit}\ (p) = \log[p/(1-p)] = \alpha + \beta' X. \qquad (1)$$

In this equation, p equaled the probability of the event to be modeled, α equaled the intercept parameter, β equaled the vector of slope parameters, and X equaled the vector of the independent variables (Agresti, 2002). A replicate factor was included in logistic regression models and models were conducted using the Proc LOGISTIC procedure in SAS (SAS Institute, 1990). This model was conducted on the percentage of parasitoids marked with yellow dye and for each of the soak and crush ELISA methods for the albumin and casein marks. Pairwise contrast tests at the 0.05 level of significance were used to separate means. When logistic regression resulted in non-significant interaction terms, interaction terms were removed and the model was re-run for each marking method. When the mean percentage (averaged across replicate cages) of marked parasitoids was equal to zero for a treatment, this treatment was removed from the logistic analysis (Agresti, 2002). Fisher's Exact tests at the 0.05 level of significance were used to compare means equal to 0 with means ≥0 (McDonald, 2008).

It was hypothesized that the use of a 'double-mark,' where parasitoids scored positively for any two of the three marks, would increase the accuracy of detecting marked parasitoids. Therefore, an additional variable indicating whether any two of the three marks were positive (referred to as a 'double-mark') was included, and the above-mentioned logistic regression analyses were conducted for this 'double-mark' variable for both the crush and soak ELISA data. Additional variables such as a 'double-protein-mark' (where a parasitoid tested positive for both proteins) and a 'triple-mark' (where parasitoids tested positive for all three marks) were not statistically analyzed due to these variables producing a lower percentage of marked *G. ashmeadi* than the 'double-mark' variable (see Results).

Multiple regression was used to determine the effect of day on the mean coverage score for the yellow dye mark and the mean ELISA optical densities for albumin and casein. This analysis was run for the soak and crush ELISA detection methods. Logistic regression was used to determine the effect of treatment on the percentage of *G. ashmeadi* containing yellow dye in their gut. This was conducted for data pertaining to the wet topical and dry residue treatments only. Quasi-complete separation of data points existed for the variables sex and day (Agresti, 2002). Therefore, sex was removed from the model and day was specified as a continuous variable. Fisher's Exact Test was used to compare the percentage of *G. ashmeadi* containing yellow dye in their gut between the dry residue and untreated controls and the wet topical and untreated controls.

Paired χ^2 tests were used to determine which method of ELISA (crush or soak), mark (yellow dye, albumin, or casein) or double-mark (for soak and crush ELISA) was more sensitive (i.e., detection of a higher proportion of marks across all treatments) for detecting the casein or albumin and which method was more accurate (i.e., detection of the highest percentage of marked *G. ashmeadi* in the wet topical and dry residue treatments and the lowest

percentage in the non-treated controls). Accuracy was measured with the following equation:

$$\text{Accuracy} = \frac{(\text{marked T \& R} + \text{unmarked C})}{(\text{marked T \& R} + \text{unmarked C} + \text{unmarked T \& R} + \text{marked C})}, \quad (2)$$

where 'marked T & R' equaled the number of marked *G. ashmeadi* in the wet topical and dry residue treatments; 'unmarked C' equaled number of unmarked *G. ashmeadi* in the untreated controls (controls); 'unmarked T & R' equaled number of unmarked *G. ashmeadi* in the wet topical and dry residue treatments; and 'marked C' equaled number of marked *G. ashmeadi* in the treatments. Paired χ^2 tests consisted of 2×2 contingency tables comparing the number of accurately accessed parasitoids [the numerator in equation (2)] with the total number of parasitoids [the dominator in equation (2)] between two marking methods.

Logistic regression was used to determine whether the percentage of *G. ashmeadi* containing the triple mark in their guts significantly varied between soak ELISA and crush ELISA. This was conducted for data pertaining to the wet topical and dry residue treatments only. For this analysis, sex was removed from the model and day was specified as a continuous variable (Agresti, 2002).

The mortality of *G. ashmeadi* was recorded daily for each replicated treatment cage. Parasitoids that were removed from each replicate every other day for detection of yellow dye and ELISA sampling were considered censored data because they were killed prematurely for analyses. For each treatment and sex, survival data were used to derive Kaplan Meier estimates of the survival functions. Log-Rank Tests of Equality at the 0.05 level of significance were used to separate survival curves across treatments and between sexes (Klein & Moeschberger, 2003).

Results

For all logistic regression analyses conducted to determine the effect of sex, day, treatment, and their interaction on percentage of marked insects for each marking method, interaction terms were not significant.

Yellow dye marking

There was no significant effect of sex ($\chi^2 = 0.88$, d.f. = 1, P = 0.35) on the percentage of *G. ashmeadi* marked with yellow dye; however, the effect of application treatment (e.g., direct topical, residue, and control) was highly significant ($\chi^2 = 118.73$, d.f. = 2, P<0.001). When data were pooled across days, overall percentage of parasitoids marked with yellow dye after exposure to the topical treatment, dry residue treatment, and untreated control was 88, 15, and 0, respectively (Figure 1). Days post-treatment had no significant effect on the percentage of *G. ashmeadi* marked with yellow dye ($\chi^2 = 5.10$, d.f. = 5, P = 0.41) and the mean coverage score of yellow dye per day ($F_{5,526} = 4.29$, P = 0.43).

Thirteen *G. ashmeadi* contained yellow dye inside their gut, which was ingested when parasitoids fed on the yellow dye mixed with casein and albumin. The percentage of parasitoids that ingested yellow dye was 2.6 and 4.5 in the residue and topical treatments, respectively. During the experiment, *G. ashmeadi* were observed drinking the triple mark in the topical treatment and scraping their mouthparts over the dried residue in the residue treatment. The percentage of parasitoids with yellow dye detected inside

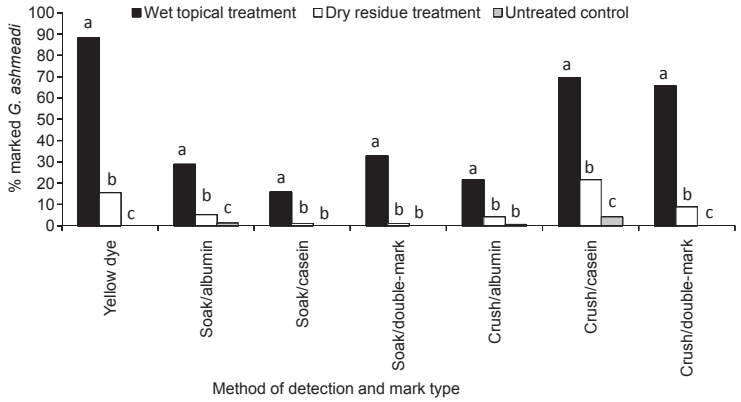

Figure 1 The percentage of *Gonatocerus ashmeadi* marked by yellow dye, casein, or albumin (the latter two marks detected by either soak or crush ELISA testing) in three treatments: (1) mark sprayed directly on parasitoids (wet topical treatment), (2) mark sprayed in experimental cages and parasitoids introduced 2 h after the mark dried (dry residue treatment), and (3) an untreated control. Different letters indicate significant differences across treatments within a marking method (logistic regression and pairwise contrast tests: P<0.05).

their gut did not significantly vary between the wet topical and dry residue treatments (χ^2 = 2.09, d.f. = 1, P = 0.15). The wet topical treatment contained significantly more *G. ashmeadi,* which had fed on the yellow dye compared with the untreated control (Fisher's Exact Test: P<0.01). The dry residue and untreated controls were statistically equivalent (Fisher's Exact Test: P = 0.06).

Albumin marking

Soak ELISA. The percentage of *G. ashmeadi* marked by albumin varied significantly by sex (χ^2 = 10.95, d.f. = 1, P<0.001) and application treatment (χ^2 = 46.53, d.f. = 2, P<0.001). Males had a significantly higher frequency of albumin marks using soak ELISA compared with females (10% of females marked; 21% of males marked). When data were pooled across days, overall percentage of parasitoids marked with albumin after exposure to the topical treatment, dry residue treatment, and untreated control was 29, 5, and 1, respectively (Figure 1). Day had no significant effect on the percentage of *G. ashmeadi* marked with albumin (χ^2 = 5.09, d.f. = 5, P = 0.40). However, the mean ELISA optical density value increased over time ($F_{5,528}$ = 2.59, P<0.05). Parasitoids that were removed from cages 9 days after the mark was applied resulted in a significantly higher (22% higher) mean optical density value compared with day 1 (Figure 2A).

Crush ELISA. Sex of parasitoids had no significant effect on the percentage of *G. ashmeadi* marked by albumin (χ^2 = 0.05, d.f. = 1, P = 0.83), whereas application treatment was significant (χ^2 = 31.17, d.f. = 2, P<0.001). When data were pooled across days, overall percentage of

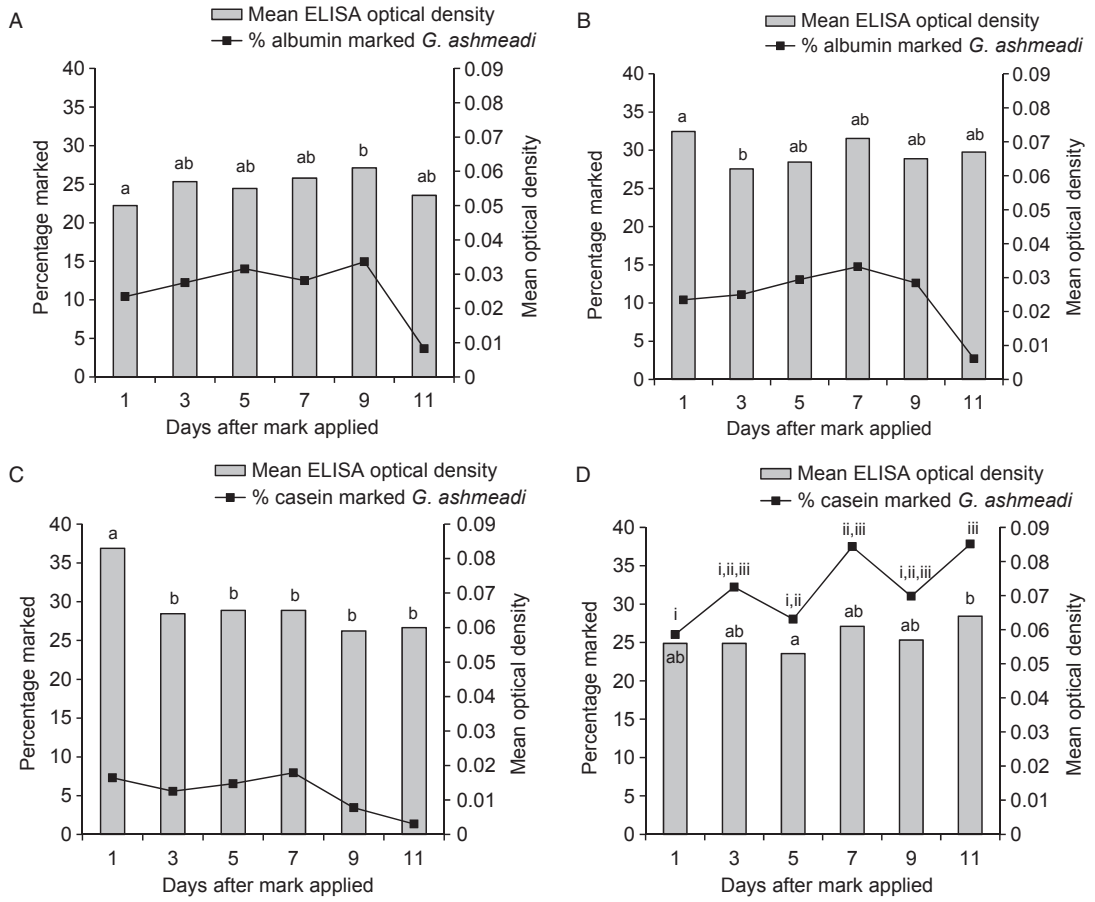

Figure 2 The effect of the number of days following application of the triple mark on the percentage of *Gonatocerus ashmeadi* marked by (A) albumin as tested with the soak ELISA method, (B) albumin as tested with crush ELISA, (C) casein as tested with soak ELISA, (D) casein as tested with crush ELISA, and the least square mean ELISA optical density [different letters (a, b, c) indicate significant (P<0.05) differences within a panel across days for ELISA optical density; different Roman numerals (i, ii, iii) indicate significant (P<0.05) differences across days for percentage of marked parasitoids].

parasitoids marked with albumin after exposure to the topical treatment, dry residue treatment, and untreated control was 24, 4, and 0.6, respectively (Figure 1). Day had no significant effect on the percentage of *G. ashmeadi* marked by albumin ($\chi^2 = 7.73$, d.f. = 5, P = 0.17), whereas the mean ELISA optical density value for parasitoids tested 1 day after the mark was applied was significantly higher (18% higher) than that measured at 3 days ($F_{5,528} = 2.70$, P<0.05) (Figure 2B).

Casein marking

Soak ELISA. There was no significant effect of sex ($\chi^2 = 0.07$, d.f. = 1, P = 0.79) on the percentage of *G. ashmeadi* marked by casein. The effect of application treatment was highly significant ($\chi^2 = 15.71$, d.f. = 2, P<0.001). When data were pooled across days, overall percentage of parasitoids marked with casein after exposure to the topical treatment, dry residue treatment and untreated control was 16, 1, and 0, respectively (Figure 1). Day had no significant effect on the percentage of *G. ashmeadi* marked by casein as detected by soak ELISA ($\chi^2 = 1.48$, d.f. = 5, P = 0.92). However, the mean ELISA optical density value for parasitoids captured 1 day after the mark was applied was significantly higher (up to 41% higher) than parasitoids tested across days 2–11 ($F_{5,528} = 8.16$, P<0.001) (Figure 2C).

Crush ELISA. Application treatment ($\chi^2 = 133.39$, d.f. = 5, P<0.001) and sex ($\chi^2 = 4.88$, d.f. = 1, P<0.05) had significant effects on the percentage of parasitoids marked with casein. Male *G. ashmeadi* had a significantly higher percentage of casein marks using crush ELISA compared with females (30% of females marked; 40% of males marked). When data were pooled across days, overall percentage of parasitoids marked with casein after exposure to the topical treatment, dry residue treatment, and untreated control was 69, 22, and 4, respectively (Figure 1). The percentage of parasitoids marked by casein ($\chi^2 = 11.79$, d.f. = 5, P<0.05) and the mean ELISA optical density values ($F_{5,528} = 2.35$, P<0.05) were both significantly effected by day. Parasitoids that were tested 7 and 11 days after the mark was applied contained 45% more *G. ashmeadi* marked by casein when compared with day 1 (Figure 2D). Similarly, the mean ELISA optical density for parasitoids tested 11 days after the mark was applied was 21% higher than parasitoids tested after 5 days (Figure 2D).

Triple marking with yellow dye, casein, and albumin

Soak ELISA. Of the 543 *G. ashmeadi* processed by soak ELISA, 63 (12%) tested positive for albumin, 30 (6%) tested positive for casein, 19 (3.5%) tested positive for both proteins, and 19 (3.5%) tested positive for all three marks. There was no significant effect of day ($\chi^2 = 5.83$, d.f. = 5, P = 0.92) and sex ($\chi^2 = 1.27$, d.f. = 1, P = 0.26) on the percentage of *G. ashmeadi* double-marked (i.e., any two marks consisting of a double combination of yellow dye, albumin, or casein). The effect of application treatment was highly significant ($\chi^2 = 27.78$, d.f. = 1, P<0.001). When data were pooled across days, overall percentage of parasitoids double-marked after exposure to the topical treatment, dry residue treatment, and untreated control was 33, 1, and 0, respectively (Figure 1).

Crush ELISA. Of the 543 *G. ashmeadi* processed by crush ELISA, 47 (8.6%) *G. ashmeadi* tested positive for albumin, 172 (32%) tested positive for casein, 41 (7.5%) tested positive for both proteins, and 35 (6.4%) tested positive for all three marks. There was no significant effect of day ($\chi^2 = 4.81$, d.f. = 5, P = 0.43) on the percentage of *G. ashmeadi* that were double-marked. The effect of sex ($\chi^2 = 4.56$, d.f. = 1, P<0.05) and application treatment ($\chi^2 = 96.20$, d.f. = 1, P<0.001) were significant. Males had a significantly higher percentage of double-marks using crush ELISA compared with females (23% of females marked; 32% of males marked). When data were pooled across days, overall percentage of parasitoids double-marked after exposure to the topical treatment, dry residue treatment, and untreated control was 66, 9, and 0, respectively (Figure 1).

Comparing marking techniques

Method of marking had a significant effect on the overall percentage (pooled across days) of marked and 'correctly detected' (as marked or unmarked) *G. ashmeadi* (Tables 1 and 2). For albumin, the overall percentage of marked *G. ashmeadi* detected was statistically equivalent between soak and crush ELISA, whereas, for casein, crush ELISA was 26% more sensitive than soak ELISA (Figure 3). Similarly, the overall percentage of 'correctly marked' parasitoids with albumin was statistically equivalent between soak and crush ELISA (Figure 3). In contrast, crush ELISA was 24% more accurate than soak ELISA for identifying *G. ashmeadi* marked with casein (Figure 3). Yellow dye and casein (tested with crush ELISA) were the most sensitive marking methods, detecting up to 29% more marked parasitoids across all treatments than the remaining marking methods (Figure 3). For crush ELISA, using a double-mark resulted in zero marked *G. ashmeadi* in the untreated controls, whereas 1–7 marked parasitoids were detected in the control when only one protein mark was considered (Figure 3). Yellow dye, casein (tested with soak ELISA), and double-mark (tested with soak ELISA) also detected unmarked parasitoids in the untreated controls.

Table 1 Matrix of pair-wise comparison tests (χ^2, P-value) comparing the effect of marking method on the overall percentage of marked *Gonatocerus ashmeadi* (for each test, d.f. = 1)

Marking method	Soak/casein	Crush/albumin	Soak/double-mark	Soak/albumin	Crush/double-mark	Crush/casein
Soak/casein						
Crush/albumin	4.4, 0.04					
Soak/double-mark	10.97, <0.001	1.78, 0.18				
Soak/albumin	12.81, <0.001	2.50, 0.11	0.08, 0.78			
Crush/double-mark	76.84, <0.001	49.47, <0.001	33.61, <0.001	30.68, <0.001		
Crush/casein	122.68, <0.001	89.41, <0.001	68.55, <0.001	64.55, <0.001	6.8, 0.01	
Yellow dye	142.28, <0.001	107.05, <0.001	84.51, <0.001	80.15, <0.001	12.92, <0.001	0.98, 0.32

Table 2 Matrix of pair-wise comparison tests (χ^2, P-value) comparing the effect of marking method on the overall percentage of 'correctly detected' as marked or unmarked *Gonatocerus ashmeadi* (for each test, d.f. = 1)

Marking method	Soak/casein	Crush/albumin	Soak/double-mark	Soak/albumin	Crush/double-mark	Crush/casein
Soak/casein						
Crush/albumin	0.88, 0.35					
Soak/double-mark	3.56, 0.06	0.91, 0.34				
Soak/albumin	3.24, 0.07	0.75, 0.39	0.01, 0.93			
Crush/double-mark	39.74, <0.001	28.97, <0.001	19.70, <0.001	20.50, <0.001		
Crush/casein	60.50, <0.001	47.12, <0.001	35.13, <0.001	36.19, <0.001	2.26, 0.13	
Yellow dye	89.09, <0.001	72.90, <0.001	57.95, <0.001	59.30, <0.001	10.44, 0.001	3.01, 0.08

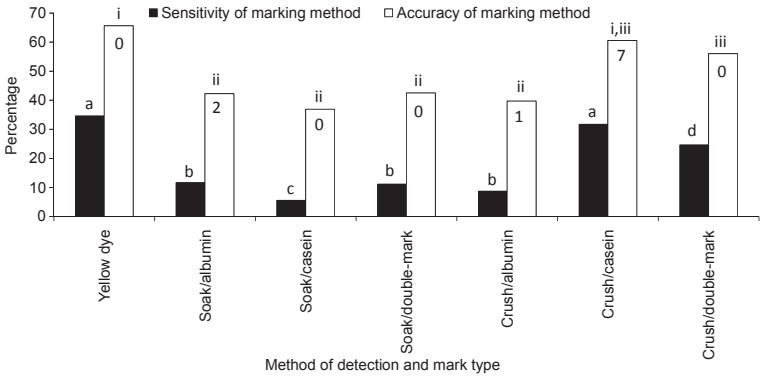

Figure 3 The overall percentage of *Gonatocerus ashmeadi* marked (sensitivity) and the percentage of 'accurately detected' marked and unmarked parasitoids (accuracy) of each marking type (yellow dye, casein, or albumin), with the latter two marks detected by either soak or crush ELISA testing method. Numbers in accuracy bar columns indicate the number of marked *G. ashmeadi* detected in the untreated controls. Different letters (a, b, c) indicate significant (P<0.05) differences across marking methods for the overall percentage of *G. ashmeadi* marked; different Roman numerals (i, ii, iii) indicate significant (P<0.05) differences across marking methods for the percentage of accurately detected tests.

When taking into consideration the number of correctly identified marked and unmarked parasitoids, the yellow dye, casein mark (tested with crush ELISA), and the double-mark system (tested with crush ELISA) were the most accurate marking methods, resulting in 29% more accuracy across all treatments compared with the remaining four marking methods (Figure 3).

For casein, crush ELISA detected 54% more *G. ashmeadi* that had ingested the triple mark compared with soak ELISA (χ^2 = 6.27, d.f. = 1, P<0.05; Table 3). For albumin,

Table 3 Comparing the number and percentage of *Gonatocerus ashmeadi* that imbibed the triple mark between soak ELISA and crush ELISA for each of the albumin and casein marks (test statistics indicate results from logistic regression analyses where d.f. = 1)

	ELISA (%)		No. detected				
Mark	Soak	Crush	Soak	Crush	No. undetected	χ^2	P
Albumin	7.7	30.8	1	4	8	2.10	0.15
Casein	15.4	69.2	2	9	2	6.27	<0.05

crush ELISA detected 23% more parasitoids that had ingested the triple mark compared with soak ELISA. However, this result was not significant ($\chi^2 = 2.10$, d.f. = 1, P = 0.15; Table 3).

Effect of application treatment and sex on parasitoid survival

Application treatment had a significant effect on the survival of *G. ashmeadi* ($\chi^2 = 56.71$, d.f. = 2, P<0.001). Specifically, the untreated control and the wet topical treatment had significantly higher mortality than the dry residue treatment (Figure 4). There was no significant difference in parasitoid mortality between the untreated control and wet topical treatment (Figure 4). In addition, sex had a significant effect on parasitoid survival ($\chi^2 = 112.72$, d.f. = 1, P<0.001) where males had a higher percentage of mortality than females (Figure 5).

Discussion

Using SARDI yellow dye to mark parasitoids

For future field studies involving marking minute Hymenoptera and where one marking method is desired, results suggest that SARDI yellow dye may have the greatest potential because this mark resulted in the highest level of marking for *G. ashmeadi*: 88% in the wet topical treatment, and no false positives in the untreated control. The use of dyes is inexpensive, and they are durable and easy to apply (Hagler & Jackson, 2001). At current prices, the use of SARDI yellow dye would cost $1 385 per ha when applied as a triple mark at a rate of 990 l ha^{-1}. Dye marking and UV visualization of the mark permit non-destructive sampling and re-sampling as part of a long-term study, which is in direct contrast to destructive ELISA testing. However, there are several potential drawbacks with using yellow dye for marking minute Hymenoptera. First, external dye may not be transferred from the larvae of ectoparasitoids as they molt or when adults emerge from pupae (Lavandero et al., 2004). Second, dried dye residue can 'flake off' and be carried by the wind into untreated plots or onto sticky traps used to collect insects during marking studies. However, the likelihood and significance of this possibility is not well known. Results from this study showed that 15% of *G. ashmeadi* acquired the yellow dye in the dry residue treatment, which is probably attributable to dried yellow dye particles flaking off the cage surface and sticking to parasitoids when they walked over the residue. Third, dusts and dyes are heavy and may negatively effect survival and behavior of minute Hymenoptera and this could cause underestimations of dispersal (Messing et al., 1993; Corbett & Rosenheim, 1996). Adverse effects on parasitoid survivorship because of exposure to yellow dye were not observed in this study or those by Schellhorn et al. (2004). Finally, a topical application of liquid dye is difficult to apply to small insects. In this study, it was observed that *G. ashmeadi* in the topical treatment were often drenched with spray and took some time to recover; therefore, minute Hymenoptera may risk drowning or being trapped and stuck to surfaces if droplets of yellow dye are not fine enough.

Using albumin and casein to mark minute parasitoids

The current study is the first to investigate the use of inexpensive albumin and casein to mark minute

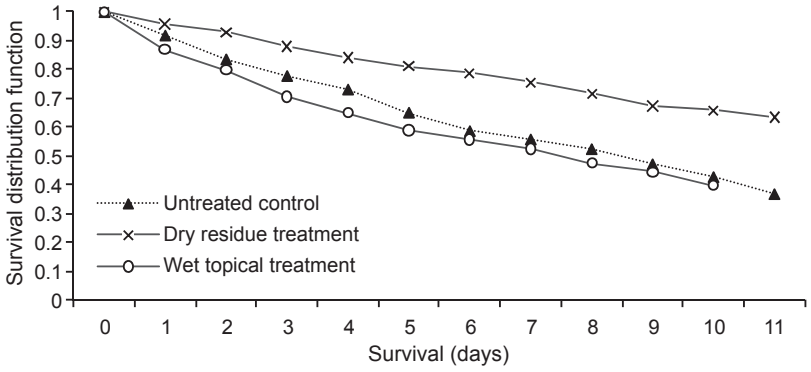

Figure 4 The Kaplan–Meier survivorship curves for effect of treatment on the combined survival of male and female *Gonatocerus ashmeadi*.

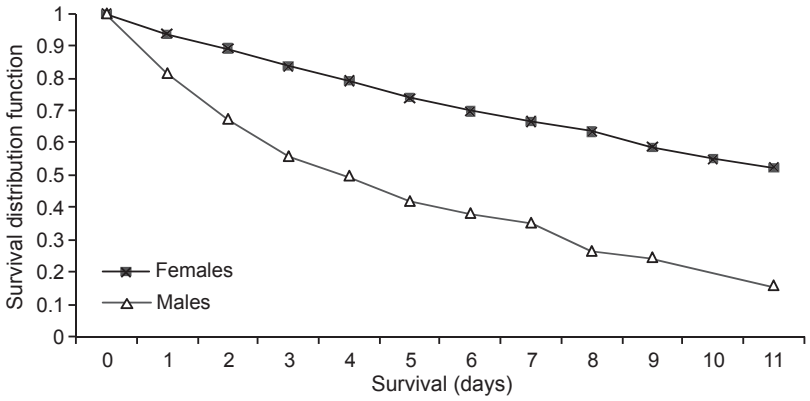

Figure 5 The Kaplan–Meier survivorship curves for effect of sex on the survival of *Gonatocerus ashmeadi* independent of experimental treatments.

Hymenoptera. Ideally, we would have preferred to just soak parasitoids during preparation for ELISA because crushing each specimen is time consuming and labor-intensive (e.g., it takes ca. 10 min per ELISA plate). However, results showed that crush ELISA was more effective than the soak ELISA.

When samples were crushed, casein alone demonstrated high potential for marking minute Hymenoptera as 69% of *G. ashmeadi* were marked in the wet topical treatment. Crush ELISA indicated that casein may be a more effective marker than albumin as casein was 23% more effective at marking *G. ashmeadi* and the marked *G. ashmeadi* detected 11 days after application increased 12% when compared with 1 day. This was not observed for albumin, where the percentage of marked *G. ashmeadi* remained statistically equivalent over time. This could be attributed to two factors. First, in the current study, 78% of the volume of spray applied was milk (containing ca. 80% casein; Anonymous 2, 2011) and 20% was chicken egg whites (containing ca. 5% albumin; Anonymous 1, 2011), so treated parasitoids were exposed to a greater volume and higher concentration of casein compared with albumin, which may have led to a higher frequency of casein marked insects as the concentration of protein marker effects marking rates (Jones et al., 2006). Second, parasitoids acquired the mark 17% more frequently when exposed to the dry residue treatment compared with untreated controls, indicating that parasitoids were able to self-mark with casein by walking over the dried protein residue. For albumin, there was no significant difference between the dry residue treatment and untreated controls, indicating that parasitoids were unable to self-mark when exposed to dried albumin residues. Consequently, the percentage of *G. ashmeadi* marked by albumin as tested with crush ELISA was equivalent over the course of the 11-day study.

The use of casein and albumin for marking minute Hymenoptera is relatively inexpensive and can be applied over a large area using any commercial spray rig (Horton et al., 2009). At current prices, casein (i.e., cow's milk) and albumin (chicken egg whites) would cost $590 per ha and $1 023 per ha, respectively, when applied as a triple mark (20% chicken egg whites, 78% of 2% reduced-fat milk, and 2% yellow SARDI dye) at a rate of 990 l ha^{-1}. This rate lies between those used in previous field studies using casein and albumin (1 380 l ha^{-1}; Jones et al., 2006) and yellow dye (600 l ha^{-1}; Schellhorn et al., 2004). For marking studies where one mark is desired, casein is ca. 50% less expensive than albumin when used at the application rates tested in this study.

Using a multi-marking system to mark parasitoids

This study combined three marks as a novel triple-marking approach in an attempt to mark as many parasitoids as possible while reducing the risk of obtaining false positives. It was hypothesized that combining proteins with yellow dye and using a double marking system would increase the percentage of marked *G. ashmeadi*. However, yellow dye was the most sensitive method, marking 88% of parasitoids in the wet topical treatment and resulted in the highest percentage of marked parasitoids across all treatments. In the current study, the triple mark was applied in the form of a combination spray; however, it is unknown whether applying the three marks separately and/or in a different form (e.g., powdered instead of liquid milk for casein marks) would improve efficiency of the double-marking system.

The inclusion of a untreated control provided a measure of 'accuracy' of the triple mark as it was assumed that the untreated controls contained no marked insects. Casein as tested with crush ELISA had a false positive rate of 1.3%. Consequently, a double-marking system using two of the three marks tested here may be imperative for studies that require very high certainty that insects have been marked. These studies demonstrated that topical double-marking

reduced the number of false positives for casein, for example, from seven to zero and resulted in 65% of treated parasitoids having a detectable mark.

Duration and mode by which parasitoids were marked

This work has demonstrated that yellow dye, albumin, and casein (tested with crush ELISA) were retained on parasitoids for the duration of the 11-day study. Hagler & Jones (2010) demonstrated that albumin was retained for at least 4 weeks on test leaves and insects sampled from treated field cages. The current study and the study of Hagler & Jones (2010) left insects in treated cages for the entire study, therefore allowing them to self mark with protein after contact with dried residues. In this study, self marking was likely as up to 17% more parasitoids acquired marks by walking over a dried residue of the yellow dye, albumin (tested with soak ELISA), casein (tested with crush ELISA), and the double-mark (tested with crush ELISA) when compared with untreated controls. The percentage of *G. ashmeadi* marked with casein (tested with crush ELISA) and the ELISA optical density for this mark increased over the duration of the study supporting that *G. ashmeadi* were able to repeatedly self mark in this treatment. Results comparing ELISA optical density values across days after marking in the current study suggest that casein (tested with soak ELISA) and albumin (tested with crush ELISA) decrease after 1–3 days, presumably because self-marking was absent.

Effect of marking on parasitoid survival

In this study, a topical application of the triple mark did not decrease survival of *G. ashmeadi* compared with the untreated control. Schellhorn et al. (2004) reported no significant effect of SARDI fluorescent dye on survival of the parasitoid *Diadegma semiclausum* Hellen. One explanation for the topical application not affecting parasitoid survival may be that the smooth cuticle and grooming behavior of *G. ashmeadi* enabled parasitoids to remove much of the dye, thereby preventing negative effects that occur from a heavy and constant coating. For *G. ashmeadi,* it was observed that the yellow dye was often present in grooves on the head, thorax, abdomen, and wing joint, indicating that dye particle distribution was patchy and retained in areas not accessible to grooming. Alternatively, it is possible that the wet topical treatment had a negative physical effect on survival of *G. ashmeadi*, but that this may have been compensated for by a positive effect on parasitoid survival through the consumption of protein in the applied marks. The dry residue treatment resulted in significantly lower mortality than the untreated controls indicating that *G. ashmeadi* in the dry residue treatment may have fed on the protein in the residue of the triple mark, which enhanced longevity (Irvin & Hoddle, 2007; Irvin et al., 2007).

The use of casein, albumin, and yellow SARDI dye shows potential for marking minute Hymenoptera. These products are readily available, inexpensive, non-toxic, and easy to apply over large areas. Detection tools are available (e.g., ELISA or fluorescent lights) for identifying and quantifying marks, are relatively inexpensive to operate, and are amenable to mechanization, which would remove human-introduced sources of error (e.g., fatigue or carelessness). Additional laboratory-based research is recommended to investigate the effect of each mark separately on insect survival, movement, and grooming behavior before larger scale field trials are attempted.

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