An Immunomarking Technique for Labeling Minute Parasitoids

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ABSTRACT A laboratory study was conducted to examine the efficacy of a novel immunomarking technique on *Anaphes iole* Girault, a minute parasitoid of *Lygus* spp. eggs. Adult *A. iole* were marked with the readily available mammal protein, rabbit immunoglobulin G (IgG), by 3 different application methods. Adult parasitoids were marked internally by feeding them a honey solution spiked with rabbit IgG and externally by contact exposure or topical mist. Marked individuals were then assayed using a sandwich enzyme-linked immunosorbent assay (ELISA) for the presence of the IgG marker using an antibody specific to rabbit IgG (antirabbit IgG developed in goat). Data indicate that the IgG marker was retained throughout the entire adult lifespan in almost (98.9%) every individual parasitoid assayed, regardless of the application method used. The advantages and limitations of using immunomarkers for mark–release–recapture studies involving minute insects are discussed.

KEY WORDS *Anaphes iole, Lygus* spp., mark–release–recapture, dispersal, parasitoid, enzyme-linked immunosorbent assay

DISPERSAL BEHAVIOR SUCH as immigration and inter- and intra-field crop movement must be precisely monitored to understand the population dynamics of insect parasitoids. Generally, parasitoid dispersal has been researched using mark–release–recapture techniques that involve marking insects with an environmentally persistent material, releasing them into the field, and recapturing them after a given time interval. A wide variety of materials have been used to mark insects for dispersal studies (Southwood 1978, Akey et al. 1991). However, marking parasitoids has proven to be a special challenge for biological control researchers because most parasitoids are very small and delicate. Conventional marking materials such as paints, dyes, dusts, and tags are ineffective on small parasitoids because they are heavy and can inhibit normal dispersal behavior (Steiner 1965).

Probably the best markers to date for studying parasitoid dispersal have been the trace elements (Jackson et al. 1988, Jackson and Debolt 1990, Akey et al. 1991). These are environmentally safe and relatively persistent in insect tissues. Studies have shown that parasitoids can be effectively marked with rubidium (Rb). For example, *Anaphes ovijentatus* (Crosby & Leonard) (=*A. iole* Girault), a *Lygus* spp. egg parasitoid, was successfully marked with Rb when reared from labeled eggs of lygus adults fed an artificial diet containing >500 ppm RbCl (Jackson et al. 1988). In a similar experiment, *Microplitis croceipes* (Cresson) was marked by rearing it in host larvae that fed on an artificial diet containing 4 different trace elements (Hopper and Woolson 1991). However, there are several drawbacks to using trace elements as biological markers. Rubidium retention in some insects declines rapidly after removal of the insect from the Rb-enriched host (Fleischer et al. 1986, Hopper and Woolson 1991) or after it feeds on a potassium–rich food (Fairbanks and Burch 1968). Some studies have reported that high levels of Rb can adversely affect the development of certain insects (Stimmann et al. 1973, Van Steenwyk et al. 1978). For example, Jackson et al. (1988) determined that *A. iole* that developed in eggs of *Lygus hesperus* Knight reared on diet containing high concentrations of RbCl (1,000 ppm) had a shorter lifespan and lower fecundity than those reared on lower concentrations of RbCl. Besides the negative effects of Rb on parasitoid biology, the detection of elemental labels is difficult. Trace element analyses require technical expertise and an expensive atomic absorption spectrophotometer. Furthermore, the analyses of samples for the presence of the trace element is extensive, time consuming, and generates hazardous waste (Akey and Burns 1991).

Recently, a simple, rapid, safe, inexpensive, and stable marking procedure was developed to mark phytophagous and predaceous insects. We externally marked adult *L. hesperus* and convergent lady beetles, *Hippodamia convergens* Guérin-Méneville, with a rabbit immunoglobulin G (IgG) solution. In the laboratory, rabbit IgG, a mammalian protein, remained detectable on *L. hesperus* for over a week after marking (Hagler et al. 1992a). In the field, rabbit IgG remained detectable on *H. convergens* for at least 4 wk under extreme temperatures (> 40°C) and rainfall (> 2.54 cm) (Hagler 1997). Because the IgG marking procedure worked so well on these insects, we planned to investigate the feasibility of immunomarking very small parasitoids.

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This study was designed to test the feasibility of labeling the minute *Lygus* spp. egg parasitoid, *A. iole,* with rabbit IgG. We examined the efficacy and retention of rabbit IgG using 3 different marking procedures. First, we marked adults internally by feeding them a honey solution spiked with rabbit IgG. Second, we marked adult *A. iole* by exposing them to a wetted filter paper containing rabbit IgG. Finally, we marked adults by spraying them with a topical mist spray. A further study was conducted to determine if protein-marked individuals can transfer the protein to unmarked individuals.

Materials and Methods

Test Insects. *Anaphes iole* were received from a commercial insectary (BioTactics, Riverside, CA) as pupae in *L. hesperus* eggs. The eggs had been removed from oviposition packets (Patana 1982) and placed onto organdy cloth held between layers of moistened paper towels to prevent the eggs from desiccating during shipment. We put the parasitized eggs into a black emergence box similar to the one described by Graham et al. (1986). Emerging parasitoids were collected in clear, lighted snapvials that were attached to the side of the box.

Parasitoid Marking Procedures. Three different methods were used to label the parasitoids: (1) ingestion (internal label), (2) contact exposure (external label) and, (3) topical contact mist (external label). Newly emerged adult parasitoids were held in cages for 1–2 h before marking. Cages were made from 33-ml snapvials with the bottom removed and screened with organdy cloth.

Ingestion. Newly emerged adult parasitoids were held without food or water for 1–2 h, then were fed a spiked honey solution containing 2.5 mg rabbit IgG per milliliter of honey (Sigma, St. Louis, MO, No. I5006). The spiked honey was streaked across the organdy screened opening of the vials, and the parasitoids were allowed to feed for 1 h before being transferred to a clean cage containing regular (unlabeled) honey. The marked parasitoids were held in an environmental chamber set at 28 ± 1.1°C, 60% RH, and a photoperiod of 14:10 (L:D) h after feeding on the spiked honey solution. Approximately 30 live parasitoids were removed daily throughout their adult lifespan (≈8 d) from the environmental chamber and frozen (−70°C). The frozen parasitoids were individually homogenized in 150 μl phosphate buffered saline (PBS) and assayed for the presence of the rabbit IgG immunomarker by the sandwich ELISA described below.

Contact. The parasitoids were allowed to feed on a regular honey solution for 1–2 h to reduce the amount of feeding on the labeled solution. A Whatman No.1 qualitative filter paper (5.5 cm) was placed in the lid of a plastic petri dish (60 by15 mm). The filter paper was soaked with 450 μl of a 5.0 mg/ml rabbit IgG solution delivered by micropipette. The parasitoids were immobilized in a freezer for 1–2 min, then tapped onto the wetted filter paper and the petri dish was covered. After a 1-h exposure (walking) on the wetted filter paper, the parasitoids were transferred into a clean cage containing regular honey and placed into the environmental chamber described above. Approximately 30 live parasitoids were removed daily (for 8 d) from the environmental chamber and frozen for later analyses. The frozen parasitoids were then homogenized in 150 μl PBS and assayed for the presence of the rabbit IgG immunomarker by the sandwich ELISA described below.

Topical Contact by Misting. Newly emerged parasitoids were held in cages for 1–2 h with regular honey, then transferred to a larger screened vial (180 ml, 49 by 110 mm). They were misted with 5.0 mg/ml rabbit IgG solution with a perfume atomizer until there was a fine mist on the screen and inside the vial. The vial was then held in an airstream until the mist had dried (≈3–5 min). After drying, the parasitoids were transferred to a clean cage containing regular honey and placed into the environmental chamber described above. Approximately 30 live parasitoids were removed daily (for 8 d) from the environmental chamber and frozen for later analyses. The frozen parasitoids were then homogenized in 150 μl PBS and assayed for the presence of the rabbit IgG immunomarker by the sandwich ELISA described below.

Cross Contamination Test. A study was conducted to determine if the protein marker transferred from marked individuals to unmarked ones. Adult males were marked by the contact marking method described above. The males were then placed into a clean cage containing regular honey and placed in the environmental chamber described above. After 24 h, the marked males were transferred to a 2nd clean vial and unmarked females were put into the vial with them. These parasitoids were removed from the cage after 24 h and frozen for later analyses. The frozen individuals were sexed, homogenized in 150 μl PBS and assayed for the presence of the rabbit IgG protein by the sandwich ELISA described below.

ELISA Procedure. A sandwich ELISA was performed on each adult *A. iole* as described by Hagler et al. (1992a). Each well of a 96-well ELISA microplate was coated with 100 μl of antirabbit IgG (developed in goat) (Sigma, No. R2004) diluted 1:500 in double-distilled H_2O and incubated overnight at 4°C (note: this incubation time can be as short as 1 h without loss of efficacy). The antirabbit IgG was discarded and 360 μl of 1% nonfat dry milk in double-distilled H_2O was added to each well for 30 min at 27°C to block any remaining nonspecific binding sites on the plates. After the nonfat milk was removed, a 150-μl aliquot of the homogenized parasitoid sample was placed in each well of the pretreated assay plate and incubated overnight at 4°C (note: in this reaction too, the incubation time can be as short as 1 h without loss of efficacy). Parasitoid samples were then discarded and each well was briefly rinsed 3 times with PBS Tween 20 (0.05%) and twice with PBS. Aliquots (50 μl) of antirabbit IgG conjugated to horseradish peroxidase (Sigma, No. A-6154) diluted to 1:1,000 in 1.0% nonfat milk, were added to each well for 1 h at 27°C. Plates were again washed as described above, and 50 μl of substrate was

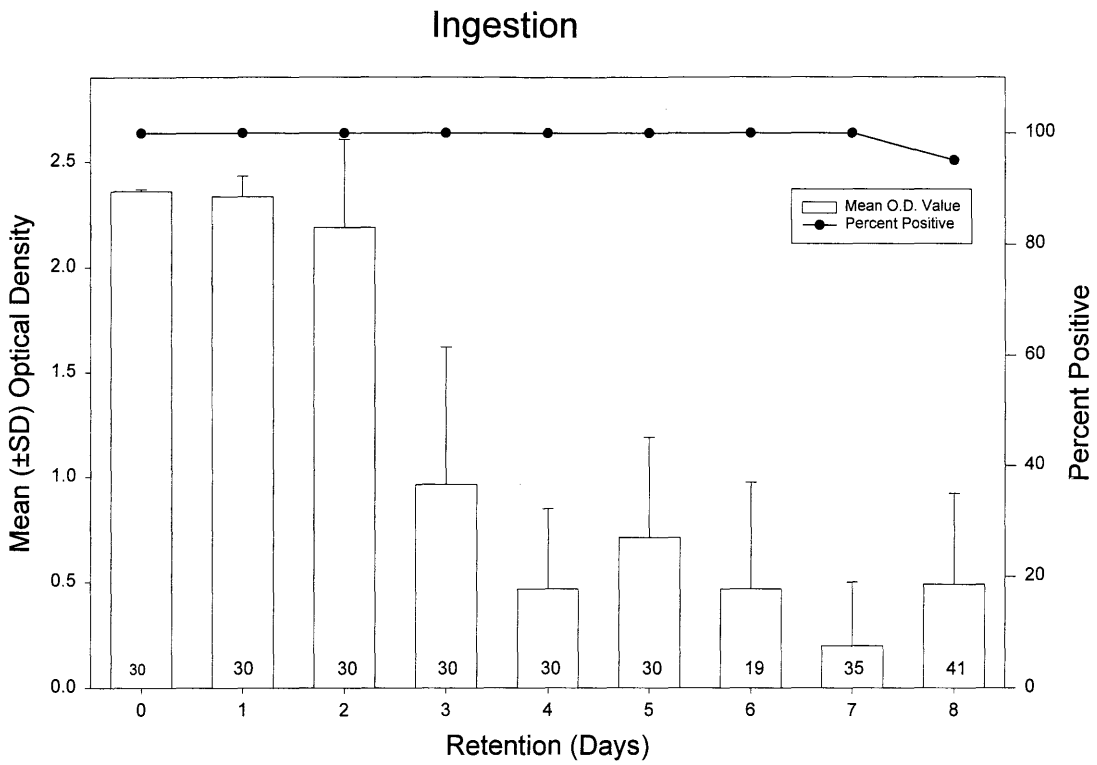

Fig. 1. The mean ± SD ELISA optical density values (left y-axis) and percentage of adult *A. iole* scoring positive (right y-axis) for the presence of rabbit IgG immunolabel after feeding on honey containing rabbit IgG. The number inside each vertical bar is the sample size for that treatment.

added using the reagents supplied in a horseradish peroxidase substrate kit (Bio-Rad, Richmond, CA, No. 172-1064). Following substrate incubation (2 h), the optical density of each sample was measured with a microplate reader set at 405 nm. The mean (±SD) ELISA optical density value and the percentage of parasitoids scoring positive for IgG remains was tallied for each date.

Negative Parasitoid Controls. Adult *A. iole* known not to contain any immunomarker were assayed by the sandwich ELISA described above (n = 262). Parasitoids known to have been exposed to rabbit IgG were scored positive for the presence of the immunomarker if the optical density value exceeded the mean negative control reading by 3 standard deviations (critical value) (Schoof et al. 1986, Hagler et al. 1992b).

Results

Retention of IgG by Ingestion. The mean (±SD) ELISA optical density values and percentage of parasitoids scoring positive for rabbit IgG remains after ingesting a honey solution containing rabbit IgG are given in Fig. 1. Mean ELISA optical density values ranged from 2.38 on day 0 to 0.20 on day 7. Virtually all of the individuals (99.3%) that fed on the spiked IgG honey solution yielded a positive immunoreaction (with exception of 2 individuals on day 8) throughout their adult lifespan. There was a considerable decrease in the amount of rabbit IgG detected in the parasitoids after 2 d; however, most of the individuals still contained easily identifiable amounts of rabbit IgG. For example, on day 7 an average immunoreaction yielded an ELISA optical density value of 0.20. This value is >100 times greater than the mean negative control value (−0.002 ± 0.01) and almost 10 times greater than the conservative (mean + 3 SD) critical value (0.028) used to score positive immunoreactions.

Retention of IgG by Contact Exposure. The mean (±SD) ELISA optical density values and percentage of individuals scoring positive for rabbit IgG after contact exposure to the wetted filter paper are given in Fig. 2. Mean ELISA optical density values ranged from 2.32 on day 0 to 0.18 on day 2. All of the individuals that walked on the IgG-soaked filter paper (with the exception of 1 individual on day 2) scored positive for IgG remains. Again, there was considerable day-to-day variation in the amount of IgG retained on the parasitiods, particularly beyond the 1-d holding interval. However, 99.6% of all individuals assayed by ELISA contained easily recognizable amounts of rabbit IgG on their body.

Retention of IgG by Topical Mist. The mean (±SD) ELISA optical density values and percentage of individuals scoring positive for rabbit IgG after receiving a topical mist spray of rabbit IgG are given in Fig. 3.

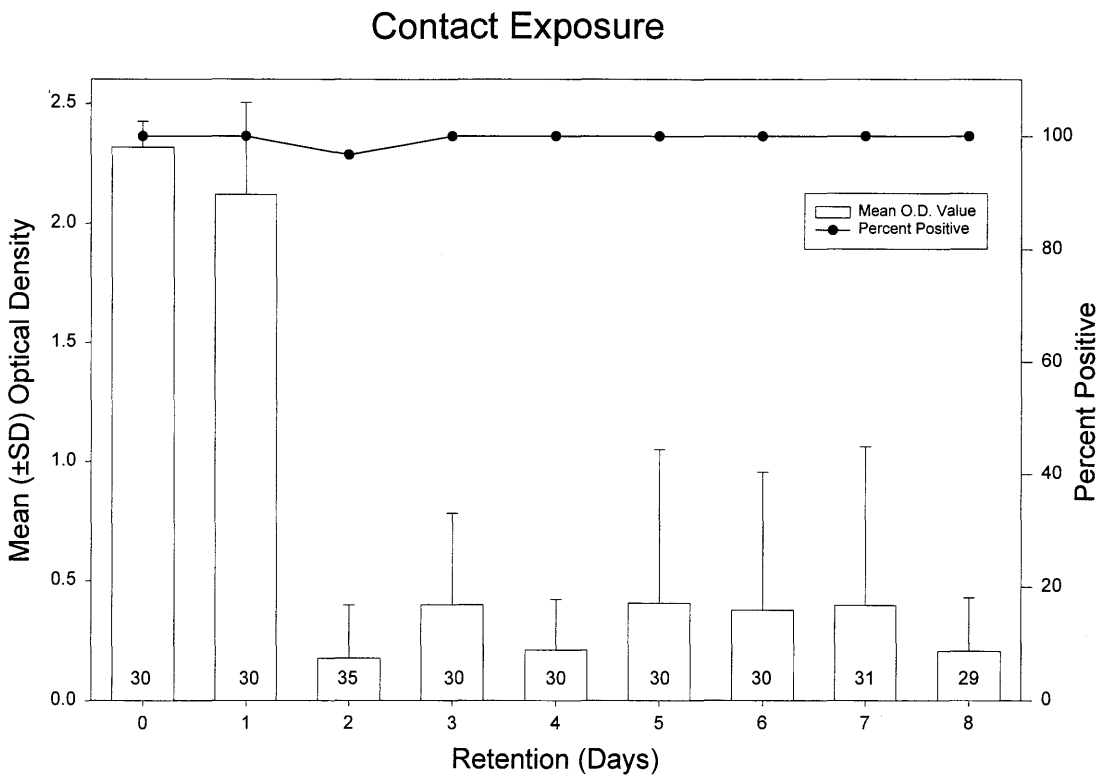

Fig. 2. The mean ± SD ELISA optical density values (left y-axis) and percentage of adult *A. iole* scoring positive (right y-axis) for the presence of rabbit IgG immunolabel after contact exposure to rabbit IgG solution. The number inside each vertical bar is the sample size for each treatment.

Mean ELISA optical density values ranged from 2.24 on day 0 to 0.19 on day 6. As with the previous marking methods, there was considerable day-to-day variation in the amount of IgG retained by the parasitoids. However, most (97.6%) of the parasitoids that were sprayed with the IgG solution yielded positive immunoreactions for the presence of rabbit IgG.

Cross Contamination Test. The mean (±SD) ELISA optical density values and percentage of males (marked) and females (unmarked) scoring positive for rabbit IgG are given in Table 1. The marked males and unmarked females yielded mean ELISA readings of 0.30 and −0.01, respectively. Most of the adult males yielded a positive ELISA response (90%) for the presence of the protein marker whereas only 1 of the females (10%) was positive (Table 1).

Discussion

Immunomarkers have been used before to label large phytophagous and predaceous insect species (Hagler et al. 1992a, Hagler 1997). Immunomarkers also have been used to label prey for predation studies (Hagler and Durand 1994). In this study we report for the first time that immunomarkers can be used to successfully mark minute parasitoids for mark–release–recapture studies. This immunomarking procedure should expedite future field studies aimed at determining parasitoid dispersal patterns. Immunomarking parasitoids offers many practical advantages over many of the more conventional marking techniques (Southwood 1978). Colored paints, dyes, and dusts are usually too big or heavy for minute parasitoids. To date, trace elements have been the most effective markers for small parasitoid species (Akey et al. 1991). However, the immunomarking technique described here offers many advantages over trace element marking. For instance, thousands of parasitoids can be quickly labeled with small quantities of the inexpensive immunolabel. The ELISA procedure is also simple, rapid, and economical. A single person with only minimal training (2 d) can assay >1,000 individual parasitoids per day. Moreover, laboratory equipment needed to run an ELISA can be found in most laboratories, and ELISA reagents are inexpensive and safe. Although an ELISA microplate reader was used to quantify ELISA optical density readings, the qualitative nature of the assay combined with the clarity of the negative control parasitoids (i.e., no background coloration) does not warrant the purchase of one.

All 3 immunomarking techniques examined in this study proved to be equally effective for labeling adult parasitoids. Overall, only 10 of the 904 parasitoids marked yielded false negative immunoreactions. This suggests that 98.9% of the individuals marked with

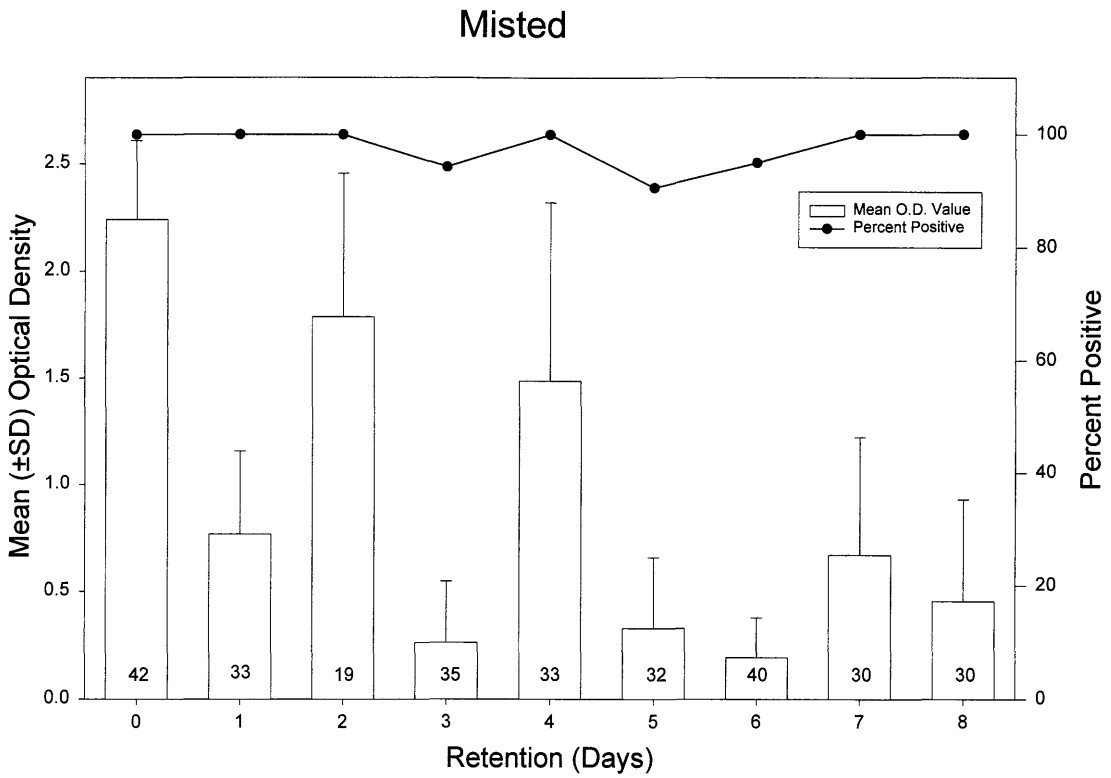

Fig. 3. The mean ± SD ELISA optical density values (left y-axis) and percentage of adult *A. iole* scoring positive (right y-axis) for the presence of rabbit IgG immunolabel after exposure to a topical mist application of rabbit IgG solution. The number inside each vertical bar is the sample size for each treatment.

rabbit IgG, regardless of the time elapsed after marking (for up to 8 d), yielded positive immunoreactions. Conversely, the negative control parasitoids yielded ELISA optical density values below zero (−0.002 ± 0.01). The low optical density values yielded by the unmarked parasitoids suggests that there is no cross reactivity between the antirabbit IgG and any of the proteins present in the adult parasitoids.

Anaphes iole proved to be an ideal model insect for this study because emerging adults readily feed on honey solution. Therefore, we were confident that most (all) of the adult parasitoids fed on the spiked honey solution shortly after emergence. There are several advantages of labeling insects by internal ingestion of foodstuffs. First, no handling of the insects is needed. This is extremely important when handling small, delicate parasitoids. Second, only a minute amount of the immunomarker is needed. We used <500 μl of honey (at 2.5 mg rabbit IgG/ml honey) for this experiment. Third, internal markers have less of a chance of effecting the normal behavior of the parasitoids. Extra care was needed for marking parasitoids by contact or topical mist. Finally, internal markers will not degrade under extreme field conditions. Internal markers are less vulnerable to washing off during rainstorms or degrading under intensive heat or humidity (see below for further discussion).

The fact that the internal marker remained detectable in the parasitoids throughout their adult lifespan (≈8 d) was surprising. It appears that rabbit IgG is slowly digested by adult parasitoids. While the amount of rabbit IgG detected generally declined overtime, the optical density values yielded 8 d after feeding were still >250 times greater than that of the mean negative control value (Fig. 1). In a previous study, we fed *Geocoris punctipes* (Say) an artificial diet spiked with rabbit IgG. These predators retained the IgG in their gut for 2.5 d when assayed by a rather insensitive

Table 1. ELISA results testing for the presence of rabbit IgG protein on unmarked females after exposure to marked males for 24 h

Treatment	*n*	Mean (± SD) ELISA value	Range, min.–max	Critical value[a]	% positive
Protein-marked males	10	0.300 (0.315)	0.003–1.029	0.011	90
Unmarked females	10	−0.010 (0.015)	−0.031–0.019	0.011	10

[a] Negative control mean ELISA reading + 3 SD.

single radial immunodiffusion assay (Hagler and Cohen 1990). Apparently, the digestive enzymes present in both *G. punctipes* and *A. iole* do not destroy the integrity of the rabbit IgG after ingestion.

Further results from this study suggest that *A. iole* also can be successfully marked externally by contact exposure. Parasitoids that were marked by contact exposure on wetted filter paper probably picked up most of the marker by tarsal or antennal contact. However, some of the individuals also were observed drinking the marking solution. While the above-mentioned internal marking procedure worked to our satisfaction on the 1st attempt, several optimization studies were needed for the contact marking method. In early pilot tests we tended to oversaturate the filter paper (i.e., >500 μl) with the rabbit IgG solution. This resulted in high parasitoid mortality from drowning. However, once the optimal volume of marking solution was determined (450 μl), the contact application method was very effective (i.e., high proportion of positive responses with minimal mortality).

The other method we examined for externally marking parasitoids was by topical spray (mist). Parasitoids marked with the topical mist probably picked up most of the marker on their dorsal surfaces. However, exposure by tarsal or antennal contact or by drinking the misted solution probably occurred even though we tried to minimize surface contact and ingestion by rapidly drying the vials in an air stream. This method also required some optimization tests before we achieved the desired results. Several spray apparatuses were tried before we used the perfume spray bottle. The perfume atomizer yielded a fine, evenly distributed spray mist. Most of the misters we tried initially yielded spray droplets that were unevenly distributed and too large. In such cases, many of the parasitoids were not exposed to the marking solution and others were damaged or killed by excessive wetting. Another misting apparatus that showed considerable promise for topically marking *A. iole* was a medical nebulizer, such as those commonly used by asthmatic patients. In a pilot test, we nebulized *A. iole* and held them for only 2 d after marking. All ($n = 66$) of those parasitoids marked with the nebulizer showed positive for the presence of rabbit IgG when assayed by ELISA (data not shown). While the perfume bottle atomizer was inexpensive and effective for marking *A. iole,* a nebulizer might be advantageous in some situations. For example, nebulizers produce an extremely fine, foglike mist that requires even less rabbit IgG solution than a more conventional mist sprayer. Furthermore, the fine mist spray decreases the chance of the marker affecting the behavior or longevity of the parasitoids and provides a more uniform distribution of the marker on the delicate parasitoids. The major disadvantage of using a nebulizer is that it is more expensive (approximately $120.00) than a cheap perfume sprayer.

The cross-contamination test showed that there is a possibility that protein–marked parasitoids can transfer the protein to unmarked parasitoids. One of the 10 unmarked females scored positive for the presence of rabbit protein. However, the ELISA reading yielded by this individual was only slightly above that of the negative control threshold (Table 1). The source of the protein transferred to this single individual is unknown. Perhaps she obtained the protein by direct contact with marked males (e.g., mating or trophallaxis), by direct contact with a vial that might have been contaminated by marked males, or by a combination of these factors. All of these potential sources for contamination are areas that deserve further investigation.

Potential limitations to immunomarking insects help identify areas for further investigation. Parasitoids may lose the IgG marker while maturing from one life stage to another. We avoided this potential problem by studying adult parasitoids. Another potential limitation is that parasitoids may lose the external marker under extreme field conditions. For example, the rabbit IgG marking protein might wash off during rainstorms or denature under high temperatures. The chances of this happening are minimized by marking the parasitoids internally by feeding them a honey solution containing rabbit IgG. Parasitoids marked by external contact or topical mist could lose their marker as a result of extreme weather conditions. However, a previous study showed that IgG proteins (rabbit IgG and chicken IgG) that were topically sprayed on *H. convergens* remained on them for >3 wk under extreme field conditions (Hagler 1997). This long retention time (i.e. >25 d) associated with immunolabels is superior to that reported for elemental markers and dusts (Van Steenwyk 1991).

Another advantageous characteristic of using immunolabels for mark–release–recapture studies is their specificity. Multiple immunomarkers can be used to mark different cohorts of individuals using different vertebrate-specific proteins. For example, in another study we determined that rabbit IgG and chicken IgG antibodies do not cross-react in a sandwich ELISA. Therefore different IgGs can be used to mark different cohorts of individuals (unpublished data).

An important area for future research includes examination of the movement of immunolabels through the food web. It has been demonstrated that rabbit IgG can be used to label insect prey for predation studies (Hagler and Durand 1994). However, it has not been demonstrated that host plants can be effectively marked with immunolabels. Levi (1970) reported that plants treated with Rb immediately absorb and translocate Rb throughout their system. Subsequently, adult lepidopteran pests that fed on Rb-treated host plants during their larval stage retained Rb as they matured (Berry et al. 1972, Stimmann 1974).

The potential sublethal effects of this mark–release–recapture technique deserve further investigation. Although we did not investigate the sublethal effects of the immunomarker in this study, it did not appear that the parasitoids were affected by the IgG marker (once we optimized the marking technique). However, studies are needed to determine if vertebrate IgGs affect parasitoid longevity, fecundity, mobility, and so on. We are initiating such studies in our laboratory.

In summary, the findings from this study suggest that rabbit IgG can be used to internally or externally mark adult *A. iole.* This immunomarking procedure and its accompanying ELISA are inexpensive (require very little specialized equipment), are environmentally safe (do not generate hazardous waste), and are technically easy (require minimal personnel training and sample preparation). The best method for applying immunomarkers will ultimately depend on the nature of the experiment, the characteristics of the parasitoid being marked, and the preference of the investigator. For *A. iole,* the best method was marking them internally by feeding them a honey solution containing rabbit IgG. However, for parasitoid species that do not readily feed on honey (or any other solution), the contact exposure or topical mist methods should work satisfactorily in most situations. Additionally, for extremely delicate parasitoid species (i.e., *Trichogramma* spp.) we recommend marking them topically with a medical nebulizer or Potter spray tower.

We expect to use this assay for further parasitoid dispersal studies. The immunoassay's specificity, sensitivity, and persistence suggests that we can label in vitro-reared parasitoids, release them in the field, and recapture them to examine their dispersal behavior. Moreover, by using multiple vertebrate-specific immunolabels, the intercrop movements of parasitoids can be examined easily. This novel immunolabeling procedure will expedite research aimed at evaluating insect pest and natural enemy dispersal patterns.

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