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Research

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Laboratory Evaluation of Egg White and Milk External Biomarkers for *Wasmannia auropunctata* (Hymenoptera: Formicidae)

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Abstract

Acquisition and retention of two protein markers were tested on little fire ants, *Wasmannia auropunctata* Roger. Pure (100%) cow's milk and a dilution (10%) of chicken egg whites were applied to *W. auropunctata* directly by contact spray plus residue or indirectly via residual contact only with protein-marked plant debris. Protein-marked ants were held in plastic shoe-box-sized containers, collected at 0, 24, and 48 h after exposure to their respective marks, and then examined for the presence of the marks by a chicken egg albumin and milk casein-specific enzyme-linked immunosorbent assay. Cross-contamination rates were assessed by allowing ants marked with egg whites to interact with an equal number marked milk for 24 and 48 h, and then collected either individually or in bulk. Results indicated that the egg white biomarker was retained longer than milk and that more ants were successfully marked when the direct spray application method was employed. Cross-contamination rates were highest among bulk-collected ants and lowest among ants collected individually after 24 h. However, the rates of cross-contamination among individually collected ants increased and were similar to that of bulk-collected ants after 48 h. On the basis of our results, external protein marking may not be suitable if mass trapping is required or if the study extends beyond 24 h due to high cross-contamination rates among specimens collected in bulk and reduced marker detection rates.

Key words: little fire ant, mark-capture, ELISA, immunomarking

The little fire ant, *Wasmannia auropunctata* Roger (Hymenoptera: Formicidae), is a major pest on the island of Hawaii and is spreading throughout the Hawaiian archipelago and the pacific region (Wetterer and Porter 2003, Vanderwoude et al. 2016, Mayron 2019). It is a very small, nondescript, and rust-colored ant approximately 1.5 mm in length. The destructive nature and mechanism by which *W. auropunctata* succeed over, and at the expense of other species, is well documented (Holway et al. 2002, Wetterer and Porter 2003, Le Breton et al. 2004). Unfortunately, despite being one of the most invasive species in the world (Lowe et al. 2000), little is known about this ant's population dynamics, foraging range, or distribution of food resources.

Wasmannia auropunctata workers forage at least as far as 6 m from their nest (Fernald 1947). However, actual distance is dependent on many factors, including humidity and terrain. No maximum foraging distance has been reported in the literature. Population densities also vary with habitat climate and food availability. The little fire ants build three-dimensional 'super-colonies' that consist of a network of small individual nest aggregations located on the ground and throughout tree canopies, between which workers move freely. To date, the only estimate of population densities was calculated by manually sorting shallow-core thatch and soil samples and counting all adult ants, brood, and larvae (Souza et al. 2008). Although this may be an accurate way to estimate populations of epigeic species, *W. auropunctata* also nest in trees and vegetation (de Souza et al. 1998), so this estimate is likely to be overly conservative.

Attempts to eradicate *W. auropunctata* have been met with varying success (Causton et al. 2005, Vanderwoude et al. 2010). A lack of knowledge of their biological and behavioral traits and the influence of environmental factors on management plans are two leading factors in poor control efficacy (Souza et al. 2008, Taniguchi 2008). Mark-release-recapture (MRR) and mark-capture (MC) techniques offer opportunities to better understand *W. auropunctata* population dynamics, nutrient flow within a colony, and spatial distribution of resources throughout an infested area. Knowledge of parameters,

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such as foraging distance and rates of trophallaxis, is vital when developing species-specific monitoring and control programs.

MRR and MC research have been integral to elucidating the behavior, dispersion, and population ecology of insects (Sunderland et al. 1995, Bowler and Benton 2005, Cordero-Rivera and Stoks 2008). Numerous marking procedures have been used with insects (e.g., physical tags, paints, inks, dyes, fluorescent dusts, trace elements, genetic markers, and proteins), but very few are useful for marking insects as tiny and delicate as *W. auropunctata* (Su et al. 1991, Evans 1997, Hagler and Jackson 2001). As social insects, behaviors such as grooming and sharing of food resources between individuals require special consideration. Care is needed to ensure that mark retention is uniform among marked individuals; there is minimal risk of cross-contamination between nestmates, and the ant's behavior is not negatively affected (Hayes 1991, Kay et al. 2010, Dickens and Brant 2014). Previous research has shown that mark retention and toxicity vary between markers applied externally and internally and among different species. Fluorescent dusts easily wash away in the rain (Rhodes et al. 1997) and are rapidly removed via grooming in social insects (Evans 1997). Internal dye markers are rapidly excreted by some termite species (Su et al. 1991). In short, finding a suitable marking technique for social insects offers another level of complexity to studying their dispersal behavior.

The methods described above have been previously tested using several ant species (Talbot 1943, Stradling 1970, Young 1980, Wojcik et al. 2000, Vega and Rust 2003); however, no studies have investigated marking techniques for *W. auropunctata*. Given their small size, the use of conventional marking techniques (e.g., topical paints, dyes, tags, etc.) is impractical, because they are likely to alter normal ant behavior (Steiner 1965). Also, preliminary observations showed that fluorescent dusts are not persistent on *W. auropunctata* (e.g., <24 h; M. Montgomery, pers. obs.).

The use of vertebrate immunoglobulin G (IgG) protein biomarkers, detectable by protein-specific enzyme-linked immunosorbent assays (ELISA) (Hagler et al. 1992) have proven useful for marking minute parasitoids (Hagler and Jackson 1998, Irvin et al. 2018) and ants (Buczkowski and Bennett 2007, Song et al. 2017, Hogg et al. 2018). However, the costs associated with IgG protein markers are prohibitive. A more cost-effective protein immunomarking technique using chicken egg whites or whole cow's milk as biomarkers was developed by Jones et al. (2006) and this second-generation marking technique has been subsequently used to mark a wide variety of insects for MC research (Hagler 2019). Protein-specific ELISA can detect protein biomarkers at minute quantities (Hagler 2019). Nevertheless, it is unknown whether biomarkers are transferred in detectable amounts between marked and unmarked individuals through ant social behaviors and specimen collection methods.

This study assesses the efficacy and suitability of using chicken egg whites (hereafter referred to as egg whites) and whole cow's milk (hereafter referred to as milk), applied directly and indirectly, as topical (external) markers for *W. auropunctata* MRR and MC research. Cross-contamination due to social interaction and collection methods (individual or bulk collections) was also examined.

Materials and Methods

The study was conducted within an enclosed rearing facility at the University of Hawaii Experimental Farm near Hilo, HI, operated by the College of Tropical Agriculture and Human Resources (CTAHR; 19°38′36.25″N, 155°84′47.89″W). *Wasmannia auropunctata* workers used in this study were obtained from laboratory colonies maintained at 26.8°C and 71% relative humidity and fed a diet of dead crickets (*Acheta domesticus*), 25% sucrose solution, and water. Experimental containers consisted of clean 35.6 × 20.3 × 12.4-cm (l × w × h) Sterilite plastic tubs (Sterilite Corporation, Townsend, MA, USA) with walls coated in Insect-a-Slip Fluon (BioQuipiProducts, Rancho Dominguez, CA, USA) to prevent from escape. Ants were sourced from stock laboratory colonies and transferred into the experimental containers using a clean, soft-bristled paintbrush before marker application. The study consisted of two components: 1) a marker retention assessment and 2) a marker cross-contamination assessment.

Marker Retention Assessment

Protein Marker Treatments

The two biomarkers tested consisted of cow's milk (Lucern Foods, Inc., Boise, ID) and ready-to-use egg whites (Lucern Foods, Inc.). The cow's milk application consisted of pure (100%) milk, and the egg white treatment consisted of 10% egg whites homogenized with water (Hagler et al. 2014). The study also contained a water only (negative control) treatment. Each biomarker was administered to cohorts of ~200 ants placed in the experimental containers described above. Two marker application methods, direct contact spray application plus residue and indirect residual contact (self-mark) application, were also examined. A water-only treatment was included to serve as negative control samples.

Acquisition of the Marks by Direct Topical Application Plus Residue

For the direct contact spray application plus residue (hereafter referred to as direct application plus residue), the ants were topically sprayed with ~1.42 ml of biomarker using a Equate hand-spray bottle (Walmart, Bentonville, AR). After the application, the ants were allowed to dry for ~0.5 h at which time a subsample of ants from each treatment was collected and labeled as the 0 h after exposure (HAE) retention treatment. All remaining ants were held in the containers in which they were treated for the duration of the experiment. Additional subsamples were collected at 24 and 48 HAE. Each ant was transferred into a 1.5-ml snap-cap micro centrifuge tube (Biologix Research Company LLC, City, ST) using a clean toothpick, then immediately frozen for later analysis. This experiment was replicated three times.

Acquisition of the Marks by Indirect Residual Contact

For the indirect residual contact mark (hereafter referred to as indirect application), the ants were placed into an experimental container that contained leaf litter that had been treated with milk or egg whites. The leaf litter was composed of *Melaleuca quinquenervia* Blake, *Eucalyptus* sp., and *Metrosideros polymorpha* Gaudich. The leaves were washed with soap and water, air dried and then treated with the respective biomarker by topically spraying the leaf litter with ~13.80 ml of the biomarker until saturated. The protein-marked leaf litter was then placed on the bottom of clean experimental containers and allowed to dry. Once dry, unmarked ants (~200 per container) were transferred from the laboratory colonies into the experimental containers. Ants from these containers were collected into individual tubes as described above at 24 and 48 HAE to the protein-marked leaf litter. This experiment was replicated three times.

Marker Cross-Contamination Assessment

A cross-contamination test was conducted by allowing ants marked with milk to interact with ants marked with egg whites. An equal number of ants treated with each biomarker via a direct application,

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as described above, were then transferred into a clean experimental container. Cohorts of ants were collected after 24 and 48 h of interacting.

The ants were collected by two different methods. Specifically, ants were collected individually, as described above, or in bulk by sweeping up multiple ants with a clean soft bristled size 3 paintbrush (Crayola, Easton, PA) into a single micro-centrifuge tube. All samples were immediately frozen for later analysis for the presence of the protein marks by ELISA.

Sample Processing

Prior to analysis, each ant sample was removed from the freezer and ants from bulk collected samples were separated into individual clean 1.5-ml microcentrifuge tubes. All ant samples were soaked in 500 µl of Tris-buffered saline for 1 h at 27°C on an orbital shaker set at 100 rpm to remove surface proteins. A 100-µl aliquot of each sample was used for the ELISA. In total, 1,592 ants were assayed for the presence of both chicken egg albumin protein found in egg whites and the bovine casein protein found in milk by the indirect ELISAs described by Jones et al. (2006).

Data Analysis

Each protein-specific ELISA plate contained at least eight negative control ant samples. Positive ELISA reactions for the presence of the egg albumin and bovine casein marks were defined as those specimens that yielded an ELISA optical density (OD) reading exceeding the critical threshold value of the mean value plus 3 SD of the negative control samples (Hagler 1997). Sample sizes for each replicate varied; therefore, data from all replicates were pooled. Descriptive statistics were calculated for all ants from the marker detectability and retention study, whereas cross-contamination rates were calculated as the percent cross-contamination among successfully marked ants only.

Results

Marker Detectability and Retention

Egg whites were more effective as a biomarker than the milk. Additionally, more of the markers were retained by the ants when applied directly than indirectly. After 24 h, the mean OD values for egg whites (0.643) and milk (0.331) applied directly were higher than indirectly applied egg whites (0.273) and milk (0.061). At 0, 24, and 48 h after direct application, egg whites were detected on 96, 98, and 98% of the individuals sampled compared with 91, 87, and 14% for milk (Fig. 1A). When applied indirectly, egg whites were detected on 76 and 12% of the ants after 24 and 48 h, respectively, compared with 18 and 2% for milk (Fig. 1B).

Cross-Contamination

The highest rate of cross-contamination was observed with 29.8% of bulk collected ants at 24 HAE (Fig. 2). The lowest rates of cross-contamination were observed with 1.9% individual ant collections, also at 24 HAE. Cross-contamination rates at 48 h were similar between the two collection methods with 17.3 and 13% cross-contamination observed among bulk and individually collected ants, respectively.

Discussion

The small size and social behavior of *W. auropunctata* limit the options for MRR and MC research. Protein immunomarking techniques have been proven reliable for tagging minute parasitoids (Hagler and Jackson 1998, Hagler et al. 2002, Irvin et al. 2018) and a wide range of social insects, including termites (Buczkowski and Bennett 2007, Baker et al. 2010), bees (DeGrandi-Hoffman and Hagler 2000, Hagler et al. 2011, Boyle et al. 2018), and ants (Buczkowski and Bennett 2007, Song et al. 2017, Hogg et al. 2018). Our study showed that direct application of egg whites was effective for topically marking *W. auropunctata* for up to 48 h. Conversely, the detectability of the milk biomarker rapidly decreased over the same period.

Acquisition and retention of egg whites and milk from the treated leaf tissue were considerably lower in this study than previously reported (Jones et al. 2006, Hagler et al. 2014). Also, the mean OD values observed for egg whites and milk at 24 HAE applied indirectly were considerably lower than the mean OD values observed in the direct application plus residue treatment for egg whites and milk 24 HAE. This suggests that the amount of marker acquired via residual transfer from the treated leaf litter was low and may have been easily removed by *W. auropunctata* through social interactions and self-grooming.

The high rate of cross-contamination among ants collected in bulk suggests that, although collecting ants *en masse* in the field is more convenient and time-efficient, it is likely to result in nearly one-third of the ants with detectable biomarkers being false-positives. The greatest potential for cross-contamination was observed mostly in relation to collection method; however, the increase in

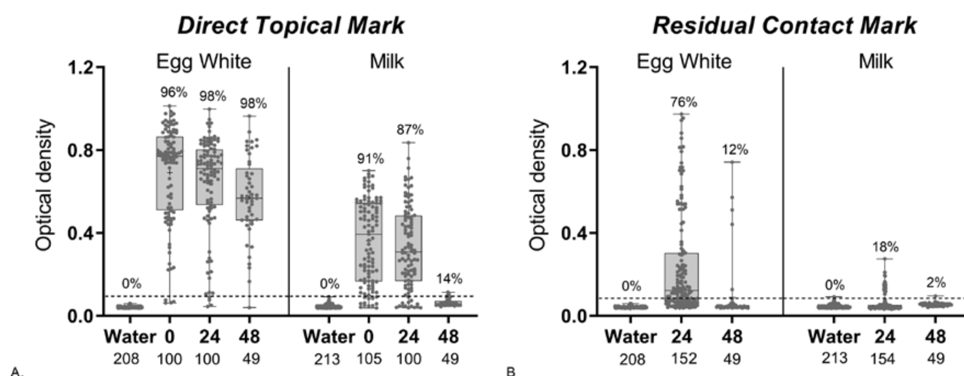

Fig. 1. Box and whisker plots showing the percent positive egg white and milk biomarker reactions for ants marked directly at 0, 24, and 48 HAE (A) or indirectly at 24 and 48 HAE (B). Dots represent individual sample OD values, and the dotted line represent the critical threshold value for a positive ELISA reaction based on the mean negative control (water only) OD value plus three standard deviations. Numbers below each *x*-axis label is the sample size for each mark treatment.

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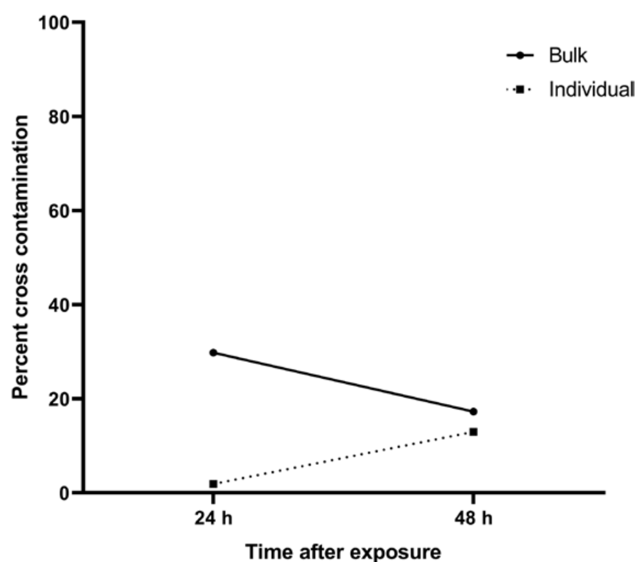

Fig. 2. Line graph showing cross-contamination rates for bulk and individually collected ants at 24 and 48 h after marker exposure.

cross-contamination rates among individually collected ants between 24 and 48 HAE suggests that biomarkers are also passed between individual ants through typical interactions.

In conclusion, selecting an appropriate technique for an ant MRR or MC study can be challenging as many biotic (social behavior, size, etc.) and abiotic factors (collection method, rainfall, temperature, etc.) can affect marker detection and retention. Our study confirms that a 10% egg white solution is retained longer than pure milk (Jones et al. 2006, Slosky et al. 2012, Lessio et al. 2014). However, for *W. auropunctata,* indirect marking by passive exposure to protein-marked leaf debris may not be reliable. Therefore, we recommend applying the marker as a direct spray to foraging trails, aggregations, and exposed nests during field studies. If mass trapping is required to collect many specimens or if the study must extend beyond 24 h, external marking, in general, may not be appropriate due low detection rates beyond 24 h and high cross-contamination rates resulting from bulk specimen collections. Although external marking may not be appropriate for *W. auropunctata* field studies, internal self-marking, whereby individuals acquire the marker by feeding on a food source laced with the marker has been used successfully for ants elsewhere (Buczkowski 2012, Hogg et al. 2018) and other insects (Rhodes et al. 1997, Hagler and Jackson 2001, Hagler and Miller 2002, Hagler et al. 2002) and may be a better marking option for *W. auropunctata*.

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