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A Nonlethal Method to Examine Non-*Apis* Bees for Mark-Capture Research

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Abstract

Studies of bee movement and activities across a landscape are important for developing an understanding of their behavior and their ability to withstand environmental stress. Recent research has shown that proteins, such as egg albumin, are effective for mass-marking bees. However, current protein mass-marking techniques require sacrificing individual bees during the data collection process. A nonlethal sampling method for protein mark-capture research is sorely needed, particularly for vulnerable, sensitive, or economically valuable species. This study describes a nonlethal sampling method, in which three non-*Apis* bee species (*Bombus bifarius* Cresson [Hymenoptera: Apidae], *Osmia lignaria* Say [Hymenoptera: Megachilidae], and *Megachile rotundata* Fabricius [Hymenoptera: Megachilidae]) were tested for a unique protein marker by immersing them momentarily in saline buffer and releasing them. Results showed that an egg albumin-specific enzyme-linked immunosorbent assay was 100% effective at detecting the protein on bees that were sampled nonlethally. Furthermore, this sampling method did not have an impact on bee survivorship, suggesting that immersing bees in buffer is a reliable and valid surrogate to traditional, destructive sampling methods for mark-capture bee studies.

Key words: dispersal, immunomarking, pollination, ELISA, egg albumin

As bee populations experience global decline (Klein et al. 2007, Koh et al. 2015), it has become increasingly important to identify and understand factors that shape their behavior in native and managed ecosystems. Large research gaps pertaining to the behavior of non-*Apis* bees, even within commercially managed species, have limited our ability to draw inferences about their success in the presence of various stressors, including urbanization, pesticide exposure, and pathogen transmission (Potts et al. 2010, Brittain and Potts 2011).

Much of the information concerning non-*Apis* bee life cycles and behaviors relies heavily upon conclusions drawn from data obtained from field observations. Such data are often made possible by labeling individuals, either prior to their release or directly in the field, with a unique mark that can be detected on specimens over time and space. Many techniques have been used to mark insects for mark-capture research purposes (reviewed in Hagler and Jackson 2001). Perhaps the most common and reliable technique for uniquely marking insects is the application of paints or dyes. Typically, these marks are applied as small dot(s) of paint on the dorsal thorax of a bee to distinguish between individuals. Paints and dyes have been routinely used to individually mark bees in enclosed semifield cage (Guédot et al. 2006, Ladurner et al. 2008, Stanley et al. 2011, Artz and Pitts-Singer 2015), laboratory and greenhouse studies (Tepedino and Torchio 1994, Birmingham et al. 2004). Another

option for individually marking bees is to glue a numbered and/or colored tag onto the thorax of each bee. Tags are frequently applied to individuals in honey bee and bumble bee colonies (Makino et al. 2006). However, tags may not be well-suited for bees with relatively smaller body sizes, or whose behavioral activities result in frequent bodily contact with abrasive surfaces that may loosen or degrade the affixed labels.

The major limitation to individually marking bees is that it is too labor-intensive for large-scale studies in which hundreds or thousands of bees are required per hectare to meet the pollination demands of agriculture. Marking an entire population of field-released bees in this way would be impractical; furthermore, the likelihood of recovery for a subset of paint-marked individuals among thousands would be limited. Collecting data for large-scale research to draw inferences about bee dispersal, foraging range, or nesting preferences would benefit from a mass-marking technique that can easily, quickly, and passively be administered to many individuals.

Current methods to mass-mark bees include the application of various colored fluorescent powders to label bee populations (Musgrave 1950, Frankie 1973, Stockhouse 1976). After application, bees are recaptured from the field, sacrificed, and examined under a microscope with UV light for the presence of colored pigments. While somewhat effective, the microscopic detection of minute

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traces of fluorescent pigments is tedious and subject to human error (J. R. H., personal observation). More recently, protein powders (such as egg albumin or bovine casein) were proven effective for mass-marking a variety of insect species, including bees (Hagler et al. 2011a,b; Boyle et al. 2018). Protein marks can be applied by a single, broadcast application (Sivakoff et al. 2012, Swezey et al. 2013, Klick et al. 2016) or by the installation of a self-marking device placed at strategic locations in the field (Hoggsette 1983, Hagler et al. 2011b, Biddinger et al. 2013). Bees can then be captured from the field, and the presence of the marks is detected on sacrificed bees by protein-specific enzyme-linked immunosorbent assay (ELISA). The application of proteins takes only a few minutes, is relatively inexpensive, and has been used effectively to draw field-based conclusions related to insect dispersal (Hagler et al. 2011b), foraging (Biddinger et al. 2013), and colonization (Hoggsette 1983). Furthermore, standardized ELISAs are well suited for mass throughput (i.e., over 2,000 individuals can be examined per day), and mark evaluations are less subject to human error. The use of ELISA to detect protein signatures on foraging bees and flowers has been implemented successfully across many bee species, including *Osmia cornuta* Latreille (Hymenoptera: Megachilidae) (Biddinger et al. 2013), *Apis mellifera* L. (Hymenoptera: Apidae) (Hagler et al. 2011a), *Megachile rotundata* Fabricius (Hymenoptera: Megachilidae) (J. R. H., unpublished data), *Bombus huntii* Greene (Hymenoptera: Apidae), *Bombus griseocollis* De Geer (Hymenoptera: Apidae); and *Bombus impatiens* Cresson (Hymenoptera: Apidae) (A. D. T. and J. P. S., unpublished data).

A notable drawback of the protein mark-capture technique is that current protocols require destructive sampling of individuals for protein analysis (Sivakoff et al. 2012, Swezey et al. 2013, Klick et al. 2016, Boyle et al. 2018). Due to the large size of honey bee colonies, the loss of a few hundred individuals for ELISA analysis will not have a major impact on a colony's welfare or to the pollination services they provide. However, this expectation does not hold for most non-*Apis* bee species, and the removal of individuals from local populations may be devastating. For example, bumble bee colonies are much smaller than honey bee colonies, typically ranging in size from as few as 20 to just over 1,800 individuals (reviewed in Cueva del Castillo et al. 2015). Thus, the removal of foraging bumblebees from a given landscape may adversely impact survivorship of both marked and unmarked colonies in the area. The potential perils of oversampling managed populations of non-*Apis* bees also hold true for solitary species, in which each nesting female is reproductively active and regularly contributes to desired pollination services during crop bloom. Simply put, for particularly vulnerable, valuable or sensitive bee species, destructive sampling for marker detection may not be an attractive option for open-field bee research.

The objective of this study was to develop a nonlethal method for examining the activity of bees marked with powdered egg albumin. Three non-*Apis* bee species, including bumble bees (*Bombus bifarius* Cresson; Hymenoptera: Apidae), blue orchard bees (*Osmia lignaria* Say; Hymenoptera: Megachilidae), and alfalfa leafcutting bees (*M. rotundata*) were selected to test this technique due to large differences in their body size, setal arrangements, grooming behaviors, and sociality, coupled with their availability for laboratory manipulations. Furthermore, these bees are widely known for their economic importance as wild and managed pollinators, and *O. lignaria* and *M. rotundata* are readily available in large quantities for experimental use. The new technique explored the immersion of protein-marked bees in individual aliquots of sample buffer and then observing the live, buffer-rinsed bees for survival over the following week. The buffer sample was then tested in the laboratory for the presence of the protein. Results were compared against

the conventional, destructive sampling technique. The methods described here provide an efficient, cost-effective and, most importantly, a nonlethal approach to sample bees for mark-capture dispersal research.

Materials and Methods

Test Insects

All three bee species were maintained in the laboratory according to generally established practices (Strange 2010, Bosch and Kemp 2001, Richards 1984, for bumble bees, *O. lignaria*, and *M. rotundata*, respectively). Three nests of *B. bifarius* were started from locally caught wild queens in May 2017. Initially, queens were individually held in small plastic cages (13 × 13 × 8 cm; Biobest, Leamington, Ontario, Canada) and provided 700 mg of beeswax-covered pollen and unlimited sugar syrup (~50% sugar solution: table sugar, water, citric acid, sorbic acid, Amino-B Booster (Honey-B-Healthy, Cumberland, MD)) and feeding stimulant (Honey-B-Healthy)). Bumble bee colonies received additional syrup and pollen was added as needed. Pollen was obtained from honey bee hives maintained in Logan, Utah. Nests were kept in darkness at 26–30°C and 40–60% relative humidity. Once each nest produced over five workers, that colony was transferred to larger plastic cages (23 × 17.5 × 10 cm; Biobest) for continuation of colony growth. Three nests were marked with protein powder after they had reached a size of approximately 100 adults (workers and males) each.

O. lignaria and *M. rotundata* were obtained as loose cocoons directly from commercial bee suppliers in June 2017 (Crown Bees, Woodinville, WA, and Hubbard and Co., Holbrook, ID, respectively) and stored in darkness at 4°C prior to emergence. Because *M. rotundata* overwinter as prepupae in alfalfa leaf-lined cocoons, they require incubation according to established best management practices to initiate adult emergence. Prepupae must be incubated at 29°C to complete development to adulthood, and female emergence occurs at about 22 d after the onset of incubation (Bitner 1976). *O. lignaria* overwinter as cocooned adults and only needed to be incubated at room temperature (approximately 22°C) to initiate adult emergence. *O. lignaria* adults naturally emerge early in the spring; due to the late time of year they were received, bees readily emerged from their cocoons on the same day they arrived.

Negative Controls

Prior to marking any bees with protein, 20 adults of each species were collected and sacrificed (lethally sampled) by placing individuals in 1.5 ml microcentrifuge tubes and freezing them at –20°C. Out of concern for any potential cross-contamination, forceps that were used for the manipulation of each bee were thoroughly cleaned after each time an individual was handled. These unmarked bees served as a negative control treatment to which positive detection was compared. The protein-treated bee samples were scored positive for the presence of egg albumin if the ELISA optical density reading exceeded the mean negative control treatment value by 6 SDs (Hagler et al. 2011a, Boyle et al. 2018).

Bee Marking, Sampling Procedures, and Survivorship Evaluations

A 1:1 (wt) mixture of egg white powder (The Barry Farm, Wapakoneta, OH) and bovine milk powder (Sigma-Aldrich, St. Louis, MO; catalog #C7078) was prepared. Bovine milk protein was included in the mixture because of its ability to adhere well to solitary bee cocoon surfaces and was deemed an effective delivery agent of the egg albumin powder (Boyle et al. 2018).

Protein powder was applied to *B. bifarius* colonies by sifting 6.6 g of the prepared mixture onto the adults and nest materials, including pupal cells. Powder was dispensed over the colonies using a fine-mesh (approximately 1 mm hole size) polyester screen (Bioquip, Rancho Dominguez, CA) stretched over a 50 ml plastic tube (Falcon, Corning, NY) containing the powder. Although trials with *O. lignaria* and *M. rotundata* were performed separately, the procedures were similar. Approximately 400 loose cocoons were gently tumbled with 5 g (for *O. lignaria*) and 3 g (for the relatively smaller cocoons of *M. rotundata*) of the powdered mixture in separate, enclosed plastic containers (22 × 12 × 12 cm). As the bees emerged, they passively marked themselves with the protein powder as they chewed through and exited their own cocoons and crawled over neighboring cocoons in the common container.

Sixty marked bees of each species were required for this study, totaling 180 individuals. Three days after their initial marking, 20 bees of each species were randomly selected to be examined for the protein mark by the conventional (lethal) sampling method. Each individual bee was placed in a sterile 1.5 ml microcentrifuge tube and frozen immediately at -20°C . Then, 1.0 ml of tris-buffered saline (TBS) buffer was added to the microcentrifuge tube 1 h before the analysis of the sample for the presence of the protein mark (see below). Another set of 20 bees of each species was randomly selected to test the nonlethal sampling method. For this treatment, an individual bee was removed from its arena (described below), placed into a sterile, sealed 5 ml centrifuge tube (Eppendorf, Hauppauge, NY) containing 1.0 ml of TBS buffer and gently agitated for 5 s. Each live bee was then removed from the centrifuge tube using clean forceps and returned to their respective holding arena (described below) and observed for survival over the following 7 d. The 1.0 ml rinsate was placed immediately in the freezer (-20°C) for later analysis by ELISA (see below). The remaining 20 protein-marked, but unrinsed, bees of each species served as controls to determine the potential effects of bee immersion in sample buffer on survival. Mortality of rinsed and unrinsed protein-marked was compared statistically using the R v.3.4.1 package “survival” to perform a log-rank test (R Core Team, 2014, Therneau, 2015) for each species evaluated.

Bee Management

Due to the social nature of bumblebees, the management of *B. bifarius* individuals varied slightly from handling methods used for *M. rotundata* and *O. lignaria*. After the colonies were marked with the protein powder, *B. bifarius* were maintained in their nest and fed pollen and sugar syrup, as described above, for 3 d. On day 3, the 20 bees that were sampled by immersion (nonlethally) were each moved into individual 90 ml plastic cups (Dart, Mason, MI) and held in an unlit incubator set at 30°C and 40–60% relative humidity and provided unlimited syrup. Similarly, an additional 20 marked bees that were not subjected to immersion were placed in cups and held under the same conditions to serve as marked, unrinsed controls. Individual cups were used to prevent intercolony aggression among individuals and for ease of assessing bee survival.

For *O. lignaria*, upon their emergence from cocoons and consequential self-marking, 60 marked female adults were collected and stored at 26°C in groups of 10 in large plastic containers (30 × 20 × 10 cm) that were replaced daily and fed a 1:1 solution of honey and distilled water. The containers were lined with a thin layer of play sand to improve bee traction to the surface of the dish and to capture protein marker residues as they potentially wore off of the bees (Boyle et al. 2018). To minimize a risk of secondary contact with the protein, bees were introduced to a new, clean container and given

fresh honey water daily. After washing the live bees in sample buffer, they were placed in groups of 10 into yet another set of containers with sand and honey water so that any bee death could be recorded. The remaining 20 marked bees, serving as marked, unrinsed controls, were also observed in groups of 10 in the containers. Mortality was then compared between the washed and unwashed bees.

M. rotundata underwent the same management and sampling design as *O. lignaria*, with minor modifications. Because *M. rotundata* are approximately one-third the size of *O. lignaria*, groups of 10 were instead introduced to sterile, 150 mm petri dishes that were replaced daily. As with *O. lignaria*, arenas were lined with sand, bees were fed a 1:1 (vol) solution of honey and water, and they were held for 3 d prior to sampling.

Protein Detection Using Anti-Egg Albumin ELISA

Each frozen, lethally sampled specimen was soaked in 1.0 ml of TBS for 1 h at 120 rpm at 27°C on an orbital shaker in preparation for ELISAs. For all TBS samples (negative control, lethally sampled and nonlethally sampled bee treatments), triplicate 100 μL aliquots of the solution were used for each assay to detect the egg albumin protein by an antialbumin ELISA described in detail by Hagler et al. (2014). All three subsamples for each specimen yielded almost the same reaction to the ELISA. As such, the data are presented as an average of each of the three ELISA readings for each specimen.

Results

The ELISA was effective at detecting egg albumin in the treated bee samples. Specifically, every protein-marked bee, whether it was lethally or nonlethally sampled, yielded a strong positive reaction for the presence of the mark (Fig. 1). Conversely, none of the unmarked, negative control bees responded to the ELISA. Because the purpose of the acquired optical density readings are to provide a benchmark for positive/negative detection thresholds, quantitative statistical comparisons between measured values were not conducted.

Survival analysis revealed no significant differences between buffer-rinsed and unrinsed bee treatments (*O. lignaria* ($\chi_1^2 = 1$, $P = 0.317$), *M. rotundata* ($\chi_1^2 = 0$, $P = 0.986$), and *B. bifarius* ($\chi_1^2 = 0$, $P = 0.986$)) over the 7-d post-rinse period that they were observed. The survival rates of rinsed bees were 100%, 95%, and 95% for *O. lignaria*, *M. rotundata*, and *B. bifarius*, respectively (versus 95%, 95%, and 95% survival for marked and unrinsed controls, respectively).

Discussion

To date, protein mark-capture studies have relied on a sampling scheme that requires that targeted insects be sacrificed for mark detection (Hagler et al. 2002, Hagler and Machtley 2016, Klick et al. 2016). This study confirms that there is potential for the use of nonlethal sampling of live individuals in the field to conduct protein mark-capture studies, with no anticipated effects of bee immersion on survival. It is likely that this technique could be applied to other bee species, considering the marker persisted on 100% of all bees tested 3 d following their initial inoculation, regardless of body size, morphology, or grooming behavior. However, the selection of other candidate species would necessarily be limited to those which are either commercially available for purchase, captured previously as solitary bee adults in cocoons, or, in the case of social bumblebees, at wild or artificial nests located at a known site. Although we did not test for persistence of the protein mark in an open-field setting, these findings have direct and positive implications for future

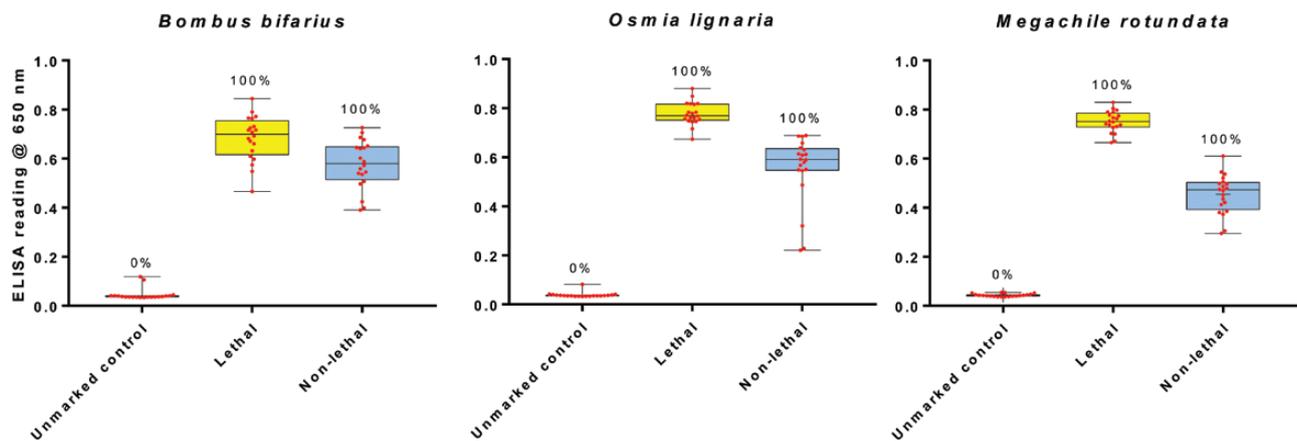


Fig. 1. Boxplot distributions of the ELISA readings and percentage (above each boxplot) of *Bombus bifarius*, *Osmia lignaria*, and *Megachile rotundata* specimens scoring positive for the presence of egg albumin. The three bee sampling treatments (x-axis) consisted of an unmarked, lethally sampled control ($n = 20$ per species); a marked and lethally sampled treatment ($n = 20$ per species); and a marked, nonlethally sampled treatment ($n = 20$ per species); respectively.

mark-capture research in which the removal of individuals from wild and/or managed habitat is a concern.

We acknowledge and emphasize that further testing would be required to verify the utility of nonlethal protein-mark sampling in an open environment, though we are confident that such an application would be successful. Boyle et al. (2018) demonstrated the durability of the protein marker in laboratory-reared, destructively sampled populations of *O. lignaria* after a successive series of buffer and water rinses of marked individuals. The protein marker persisted on rinsed specimens 18 d following *O. lignaria* emergence, suggesting that exposure to environmental forces such as adverse weather and in-field irrigation would not contribute to degradation of the marker on free-foraging bees.

Several examples portray the potential utility of this technique. Bumble bees have been widely implemented for U.S. greenhouse pollination of tomatoes and peppers since commercial colonies first became available in the 1990s (Velthuis and van Doorn 2006). Commercial bumble bee use has been correlated with pathogen spillover to wild colonies in the vicinities of greenhouses (Colla et al. 2006, Otterstater and Thomson 2008). However, the degree to which greenhouse-confined bumble bees escape and interact with wild populations has not yet been adequately characterized. Understanding the frequency and movement of greenhouse-confined bees throughout the local environment is of critical importance to the bumble bee industry. Bumble bees are also of conservation concern, with several species in decline worldwide, and one species, *B. affinis*, recently listed for protection under the U.S. Endangered Species Act (Arbetman et al. 2017, Christopher 2017). Because bumble bees are relatively easy to rear in captivity, reintroduction programs for *B. affinis* could become part of recovery management for this endangered species. The potential drivers of bumble bee decline are numerous and poorly understood (Goulson et al. 2015). As such, a nonlethal method for tracking the movement of bumble bees is of utmost importance. This study relied on *B. bifarius* to test the practice of immersing bumble bees in buffer for nonlethal mark-capture research. While this native species is not currently commercially available, it is ecologically important as one of the most widespread and abundant bees of western North America (Lozier et al. 2013, Koch et al. 2015). Additionally, this species serves as an appropriate surrogate for other bumblebees, due to similarities across *Bombus* spp. morphology and behavior.

O. lignaria is a solitary bee species managed for commercial pollination of tree fruit orchards including apple, cherry, and almonds.

Current management practices suggest use of only 618–680 females per hectare to achieve effective crop pollination in most orchard settings (Bosch and Kemp 2001). Furthermore, the establishment and reproductive success of managed populations are generally poor, which makes the acquisition of *O. lignaria* for commercial pollination expensive (ca. \$1.50 USD/female; J. Watts, personal communication). The low stocking rate and high cost of *O. lignaria* makes conventional, destructive sampling of marked individuals particularly unattractive for use in research studies that seek to improve management practices.

M. rotundata are readily available and relatively inexpensive (ca. \$0.01 USD/cocoon; J. Watts, personal communication) bees that are frequently purchased for the commercial pollination of seed crops, such as alfalfa and canola. However, understanding their behavior and dispersal in agroecosystems is still critical for improving bee management and safe-guarding populations in commercial crop production. Furthermore, the deregulation of genetically engineered alfalfa varieties (such as glyphosate-resistant and low lignin varieties) in the United States has elicited concerns over pollinator-mediated movement of transgenic pollen into neighboring conventional (non-transgenic) alfalfa seed fields (Hagler et al. 2011b; AOSCA, 2012). The practice of marking emerging bees with egg albumin provides an important tool for measuring how the dispersal and foraging behaviors of *M. rotundata* could contribute to undesired pollen flow across alfalfa fields managed both for seed production and forage.

Although we are confident in this marking technique, we acknowledge the possibility that the protein mark could have transferred between grouped individuals of both *O. lignaria* and *M. rotundata*, within their arenas, over the 3 d prior to sampling. However, this is unlikely, because arenas contained only female bees that seldom interact through close contact (e.g., no mating attempts, fighting, or sharing of nesting substrates), and no interactions were observed or documented for either species during this study. In previous dispersal research, it was apparent that the risk of netting multiple marked bees in the same net did not result in extraneous transfer of protein materials among individuals (Hagler et al. 2015). This outcome suggests a minimal crossover of protein powder between individual solitary bees in the current study, as one could expect a similar level of contact among individuals within arenas. For social bumble bees, proteins were certainly transferred among individuals and to newly emerging adults within each nest after treatment. This could be seen as a benefit for studies tracking the movement of bees throughout

the landscape by using treated nests as known protein sources. Both mature foragers and newly emerged adults would likely be exposed to proteins repeatedly through nest interactions and contact with residues over the course of the season (DeGrandi-Hoffman and Hagler 2000). Thus, studies using this method should be aware of the likelihood of within-nest transfer.

For researchers interested in implementing this method in-field studies, we recommend paint-marking immersed individuals to eradicate any concern over duplicate sampling over time; the present study did not address whether the protein could be detected after successive rounds of introducing bees to the buffer. This, and an evaluation of any resultant effects of immersion in buffer to in-field bee foraging or dispersal behavior are areas that may warrant further evaluation. Provided the results obtained from Boyle et al. 2018, it would be likely that secondary or tertiary immersions of the same marked bee would result in the positive detection of egg albumin. Regardless, we show that a protein mark can be detected with 100% efficiency on nonlethally sampled bees. Furthermore, this mass-marking technique would greatly enhance rates of recapture of marked bees in an open environment. The ease, consistency, and low cost of powder-marking provides means for evaluating bee dispersal on the scale of hundreds to thousands of individuals. This technique can be used as a harmless means for in-the-field sampling of live bees for future protein mark-capture research. This method will be especially useful for studying the dispersal patterns of sensitive or valuable species. While techniques for application may vary depending upon specific management practices, or the species to be evaluated, it is likely that this simple, effective, and nonlethal technique can be expanded to study many other arthropods, including both pollinating and nonpollinating species.

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