

Behavioral Effects and Retention of Protein Immunomarkers on Plum Curculio *Conotrachelus nenuphar* (Coleoptera: Curculionidae)

Timothy Lampasona,^{1,4,*} Angelita Acebes-Doria,^{2,*} Tracy C. Leskey,³
and Anne L. Nielsen^{1,*}

¹Department of Entomology, Rutgers University, Bridgeton, NJ, USA, ²DKI US Pacific Basin Agricultural Research Center, USDA Agricultural Research Service, 64 Nowelo St, Hilo HI 96720, USA, ³Appalachian Tree Fruit Research Center, USDA ARS, Kearneysville, WV, USA, and ⁴Corresponding author, e-mail: tpl41@rutgers.edu

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Abstract

Protein immunomarking can be used to track the dispersal of insects in the field or identify plant–insect interactions. By marking insects with known proteins and recapturing them, their movement or host use can be quantified with Enzyme Linked Immunosorbent Assay (ELISA). Before using this technique, retention and behavioral effects of these markers should be evaluated to ensure that the insect's natural behaviors are conserved. Here, we tested the effects of protein markers on the plum curculio, *Conotrachelus nenuphar* (Coleoptera: Curculionidae) using two different application methods. This weevil is native to North America and a pest of tree fruit and blueberry in the United States and causes damage resulting in near complete crop loss if left untreated. We tested the effects of marking adult *C. nenuphar* with two inexpensive food-based immunoprotein markers, bovine casein (cow's milk) and chicken albumin (egg whites) on climbing distance (total cm), lateral movement (total cm), and lateral movement speed (cm/s), as well as retention time of protein immunomarkers. Neither protein immunomarker affected *C. nenuphar* movement or climbing, although females climbed significantly greater distances than males. ELISA assays detected 37.5–56.2% of milk protein and 56.2–59.3% of egg on the insect 7 d after application depending on application method. Our findings indicate that food-based protein immunomarkers can be used in future studies to test *C. nenuphar* movement within host plants without impacting behavior. The use of protein immunomarking will allow studies that will lead to behaviorally based management tactics.

Key words: insect marking, EthoVision, dispersal, weevil, mark–capture

The dispersal patterns of insects, while once largely ignored, have become vital pieces in both insect ecology and integrated pest management (IPM) (Stinner et al. 1983). Understanding movement within an agroecosystem can allow management professionals to target insects during vital stages in the growing period (Chouinard et al. 1992, Leskey et al. 2020, Stinner et al. 1983). This avoids damage to a crop, and potentially decreases insecticide applications, as applications can be spatially refined to better match insect distribution patterns (Blaauw et al. 2016, Klick et al. 2016). Considering that insects are our greatest competitors for plant-based food, a clear understanding of the movement and dispersal within or between crops is vital to any IPM program (Irwin 1999).

Studies of insect dispersal often rely on marking insects for recapture and delineation of movement patterns. In other studies, methods such as fluorescent powders (Reid and Reid 2008), rare elements (Moss and Van Steenwyk 1982), radioisotopes (Berry et al. 1972),

dyes (Hendricks and Graham 1970), and mutilation have been used (Murdoch 1963). The main drawback with these methods is that they are largely only capable of marking captive insects which are released for later recapture, making them reliant on large captive populations to be informative. Additionally, they may affect insect behavior or survival depending on the marking method and insect species (Reid and Reid 2008). Another method used to study insect movement when direct observation is impractical is the use of protein immunomarkers. This method utilizes inexpensive animal proteins to mark organisms, rather than pigments, radioisotopes, or tags (Hagler and Machtley 2016). The main benefit of protein marking over these other methods is that proteins can be applied en masse, and can be applied to the environment allowing for passive contact with insects in situ. The use of proteins to mark insects has evolved in their efficacy and affordability over time, from the relatively expensive rabbit immunoglobulin G (IgG), which last on insects for up to a month (Hagler et al. 1992;

Hagler 1997; Hagler and Miller 2002; Baker et al. 2010) to food-based proteins, a method developed by Jones et al. (2006) and later field tested on a diverse array of insects by Hagler and Jones (2010). The food-based materials are much less expensive than previous proteins, which could cost as much as \$500/L (Hagler and Jones 2010, Biddinger et al. 2013). In the present study, we evaluate two food-based protein immunomarkers for impacts on behavior and the longevity and degradation curve of these materials on *Conotrachelus nenuphar* Herbst (Coleoptera: Curculionidae) a pest of tree fruit and blueberry (Rydb.) (Ericales: Ericaceae) in the Eastern United States (Lampasona et al. 2020). This insect was chosen because of our plans to use these methods in field trials, but similar methods can be utilized to determine the efficacy of protein marking on other insect species.

C. nenuphar dispersal is difficult to study as their small size (3.5–6mm), nocturnal behavior, and thanatosis response to disturbance makes direct observation difficult in the field (Quaintance and Jenne 1912, Racette et al. 1991). Thus, in situ marking of *C. nenuphar* with protein immunomarkers would be an ideal method to study their dispersal. To study this marking method as a tool to track movement of *C. nenuphar*, we tested the behavioral effects and retention time of two common protein markers, bovine casein and egg albumin. We used the visual motion tracking software EthoVision to measure the movement of *C. nenuphar* marked with each protein via residual activity on a substrate or direct spray. Additionally, we used Enzyme Linked Immunosorbent Assay (ELISA) protocols developed by Jones et al. (2006) to test protein retention over time.

Materials and Methods

Behavioral Analysis of Marked *Conotrachelus nenuphar*

Insect movement was measured similarly to protocols in Lee et al. (2014). Adult *C. nenuphar* were grouped by sex ($n = 60$ each) and divided evenly among the treatments and protein types. All protein mixtures consisted of the protein (5% egg albumin or 20% bovine casein), water, 0.03 g/L of ethylene diamine tetra acetic acid (EDTA), and 1300 PPM Silwet spreader/sticker. Adult *C. nenuphar* were ‘marked’ with either the 5% egg albumin, 20% bovine casein mixtures, or a water control. To represent the different ways that insects may pick up proteins, two application methods were compared: proteins were sprayed directly onto the insects via a spray bottle (‘direct’) or sprayed onto a plastic 60 mm Petri dish which was dried for 2 h prior to introduction of the insects (‘residual’). For direct application, insects were placed in a paper cup, and given two full sprays from an 8 oz spray bottle. Insects were then turned out into an untreated 60 mm plastic petri dish and given 2 h to dry. For residual application, Petri dishes were sprayed twice with the same spray bottle as in the direct application. Untreated insects were introduced to the dishes for 2 h before being removed for testing. All insects were evaluated in groups of five at a time. Adult age and mating status were not known.

Marked *C. nenuphar* adults were then placed in 60 mm glass Petri dishes on a light board and allowed to move for 60 min. *C. nenuphar* movement was filmed and analyzed using EthoVision software (version 3.1.16, Noldus Information Technologies, The Netherlands), set up in a dark room at ~25°C. Total movement distance (cm), and velocity (cm/s) were measured. After the movement test, insects were placed at the bottom of clear polycarbonate cylinders (7 cm diameter; 30 cm tall). Insects were assigned to random arenas under fluorescent lights (~5,400 lux) at ~25°C. Each insect was allowed 25 min to climb, and the total height climbed (cm) was recorded.

As insects reached the top of the cylinder, it was flipped, and insects were able to continue climbing towards the newly oriented top (Lee et al. 2014). Containers were flipped carefully to avoid dislodging insects, which could elicit a thanatosis response. Insects were then placed in rearing containers inside incubators held at 25°C, in 16:8 L:D, with apples and water. Containers were checked for mortality at 24 and 48 h postapplication and then sexed. All insects used in this experiment were laboratory reared at 25°C, in 16:8 L:D, 70% RH, in USDA-ARS laboratory facilities in Kearneysville, WV, USA.

Retention of Protein Markers

C. nenuphar was collected in Spring 2019 from unsprayed peach (L) (Rosales: Rosaceae) orchards at the Rutgers Agricultural Research and Extension Center (RAREC) in Bridgeton NJ, and kept at 25°C, 16:8 L:D, 70% RH, until experimentation began. Age and mating status were not controlled, but insects were collected from the same cohort of foraging adults by use of beat sheets and unbaited trunk traps. *C. nenuphar* were divided by sex and treated with either direct or residual method as above. Groups of five insects were removed from Petri dishes and placed in 946 ml plastic deli containers with five thinning apples and a water wick. At periods of 1, 3, 5, and 7 d post protein contact, 4 insects each, 2 males and 2 females were removed from containers for each protein type and application method and placed in separate microcentrifuge tubes and frozen at -20°C for ELISA analysis (i.e., at day 5, two males and two females were removed from each protein type/application method combination, for a total of 16 insects removed per day). At experiment end, a total of 120 *C. nenuphar* were analyzed, while unmarked insects were used as negative controls in the ELISA analysis.

Two ELISA tests were performed, following methods from (Blaauw et al. 2016) modified from methods described by Hagler and Jones (2010), to detect for the presence of egg white or bovine casein protein on *C. nenuphar*. We used commercially available antibodies for bovine casein and chicken egg albumin, rabbit anti-casein (bs-0813R; Bioss Inc., Woburn, MA), and rabbit anti-egg (C6534, Sigma–Aldrich, St. Louis, MO) respectively. Peroxidase conjugated secondary antibody was used for both egg and milk (31503; Pierce Biotechnology, Rockford, IL) donkey, anti-rabbit IgG (H + L) (SAB3700926; Sigma–Aldrich).

One ml of the extraction buffer solution, tris-buffered saline (TBS, pH 8.0; T6664; Sigma–Aldrich) plus 0.3 g/liter EDTA, was added to each microcentrifuge tube containing *C. nenuphar* insect samples. Samples were soaked in this solution for 3 min, while being disturbed to immerse samples in liquid. An 80 µl aliquot of each sample was pipetted into individual wells of a 96-well microplate (Nunc-Immuno MaxiSorp; Thermo Fisher Scientific). Each 8 × 12 microplate was laid out with the first two columns as unmarked *C. nenuphar* controls, the next eight with samples, the 11th column as extraction buffer only, and the last as positive controls (5% egg or 20% milk solution, as described above). Samples were incubated at 37°C for 2 h on an orbital plate shaker (Standard Orbital Shaker, Model 3500, VWR International), after which all well contents were discarded.

For both protein assays, microplates were washed three times with 300 µl/well phosphate buffered saline (PBS; P4417; Sigma–Aldrich) plus 0.09% Triton-X100 (X100; Sigma–Aldrich) (PBST). After washing, 300 µl/well of blocker solution was added to each well. Blocker was composed of PBS + 1,300 Silwet L-77 (Momentive Performance Materials Inc., Columbus, OH) plus 20% bovine serum (B-9433; Sigma–Aldrich) for egg white assays or 10% ethanolamine (E9508; Sigma–Aldrich) for the milk assays. Plates were placed back on the rocker for 1 h of blocking, after which they were washed

twice with 300 μ l/well of the PBST solution. Then, primary antibodies were used, diluted at a ratio of 1:6,000 for egg white assay, and 1:1,000 for milk assay in a solution of PBS plus 1,300 ppm Silwet L-77 and 20% bovine serum. Secondary antibodies were diluted in the same solution, but at a ratio of 1:28,000 for both egg white and milk assays. Antibody solutions were determined via the use of a checkerboard titration assay (Crowther 2001). After introduction of primary antibodies, plates were incubated for 30 min, and contents then discarded. After being washed twice with the 300 μ l/well PBST, 80 μ l/well of secondary antibody solution was added, and then were allowed to incubate for 2 h, then discarded.

Microplates were washed three times with 300 μ l/well PBS plus 2.3 g/liter sodium dodecyl sulfate (SDS; L-4509; Sigma-Aldrich) (PBS-SDS), followed by three washes with 300 μ l/well PBST. Then, 80 μ l/well of Ultra-TMB substrate solution (34028; Thermo Fisher Scientific) was added to each well, and the plates were incubated in the dark, on the plate shaker at room temperature for 10 min for the milk assay and 5 min for the egg white assay. Afterwards, 80 μ l/well of 2 N H₂SO₄ (258105; Sigma-Aldrich) was added to stop any reaction.

A BioTek 800 TS Absorbance Reader (BioTek Instruments, Inc., Winooski, VT) measured the optical density (OD) for each sample at 450 and 490 nm. The mean of these numbers is used to determine if a sample is marked or unmarked, and a higher OD score means that a higher concentration of marking material has been picked up in the assay.

Data Analysis

For each behavioral metric (distance moved, distance climbed, and movement speed) data did not meet assumptions of normality and were analyzed as a full factorial design, with application method, protein type, and sex as factors in a Generalized Linear Model with Poisson distribution.

Presence or absence of protein, based on the average OD score, relative to the unmarked control samples was calculated according to Sivakoff et al. (2011). Percent insects marked per replicate to examine retention time were transformed using ArcSin(sqrt(x)) and analyzed using repeated measures analysis of variance (ANOVA). Days since application and application method were used as factors in a 2-way ANOVA. Sex was initially treated as a factor but was excluded from the final analysis after no significance was observed. Posthoc analysis was performed using Tukey's HSD to separate means. All analysis was performed using JMP Pro 15 (SAS, Cary, NC).

Results

Behavioral Analysis of Marked *C. nenuphar*

There were no significant differences in *C. nenuphar*'s climbing or lateral movement between direct spray or residual exposure to protein, or between different protein types compared with an unmarked control (Fig. 2). No mortality of control or marked insects occurred within 48 h of application. Female *C. nenuphar* climbed significantly higher distance than males ($\chi^2 = 13.808$, $df = 1$, $P = 0.0002$), but climbing distance was unaffected by protein type ($\chi^2 = 3.535$, $df = 2$, $P = 0.171$) or application method ($\chi^2 = 0.189$, $df = 1$, $P = 0.664$).

Protein type and application method were not significant factors in lateral movement (type: $\chi^2 = 2.7479$, $df = 2$, $P = 0.2531$; method: $\chi^2 = 0.0308$, $df = 1$, $P = 0.8607$; Fig. 3) or speed (type: $\chi^2 = 2.3119$, $df = 2$, $P = 0.3148$; method: $\chi^2 = 0.3221$, $df = 1$, $P = 0.5704$; Fig. 4). Sex was not a significant factor in lateral movement ($\chi^2 = 0.0308$, $df = 1$, $P = 0.8607$) or speed ($\chi^2 = 0.3221$, $df = 1$, $P = 0.5704$).

Protein Retention OverTime on *C. nenuphar*

Protein retention was high and decreased significantly by the seventh day ($F = 5.3682$; $df = 4,79$; $P = 0.0008$, Fig. 1a). For all milk protein data there was no interaction observed between application method and days since application ($F = 0.5912$; $df = 4,79$; $P = 0.6701$). Milk protein retention was identical between residual and direct applications ($F = 0.000$; $df = 1,79$; $P = 1$). The whole model was significant for milk protein ($F = 2.6486$; $df = 9,79$; $P = 0.0106$).

Similarly, retention of egg protein was high through the seventh day since application ($F = 9.2257$; $df = 4,79$; $P \leq 0.0001$, Fig. 1b). There was no interaction between application method and days since application ($F = 0.2888$; $df = 4,79$; $P = 0.8843$). Application method alone was also not a significant factor in retention ($F = 1.2233$; $df = 1,79$; $P = 0.2725$). Optical Density (OD) scores reported below (Table 1). The whole model was significant for egg protein ($F = 4.3646$; $df = 9,79$; $P = 0.0002$).

Discussion

We found that protein immunomarkers are an effective marking method for *C. nenuphar* and do not negatively impact the behaviors we measured that are important for dispersal. We also found that protein immunomarkers have high retention rates up to 7 d postapplication under laboratory conditions, and while milk had numerically lower retention earlier in the week, both milk and egg had high average retention over a 7-day period, with their lowest retention at day 7 after

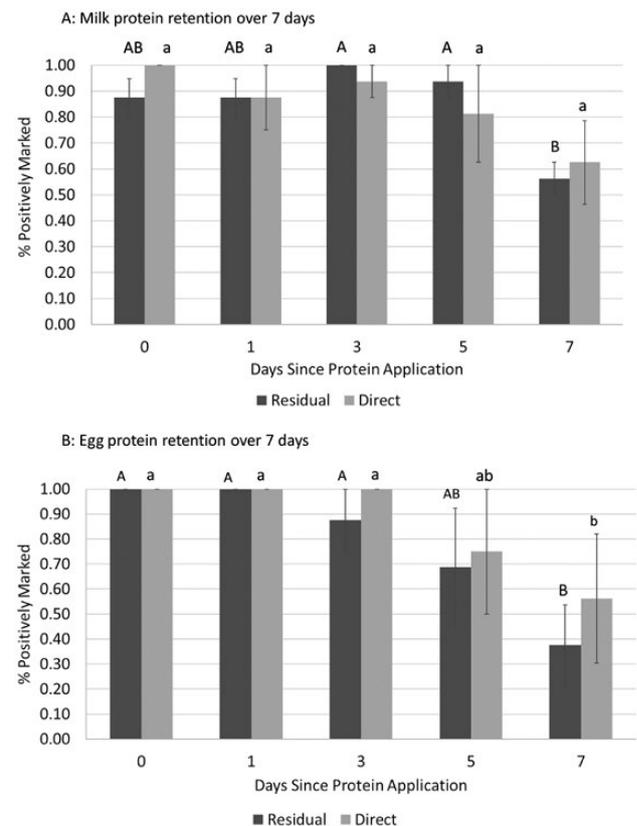


Fig. 1. a) Egg protein degradation. b) Milk protein degradation. Retention over time measured as percent of insects positively marked at different time points postapplication (mean \pm SEM). Separated by application method (residual or direct). Letters indicate significant difference between retention over days sprayed ($P < 0.05$).

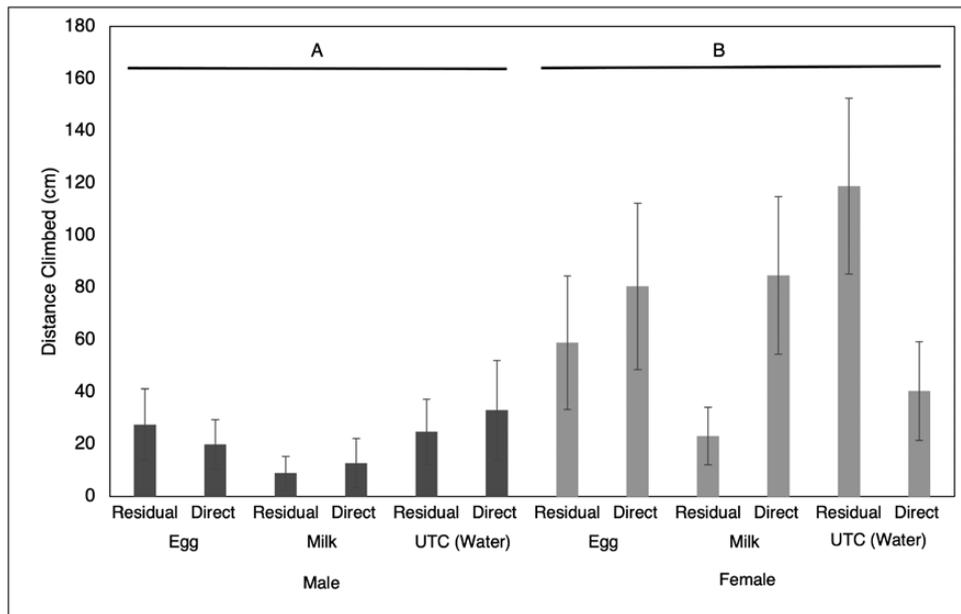


Fig. 2. *Conotrachelus nenuphar* climbing distance. Only sex was a significant factor in total distance climbed ($\chi^2 = 13.809$, $df = 1$, $P < 0.001$) (mean \pm SEM).

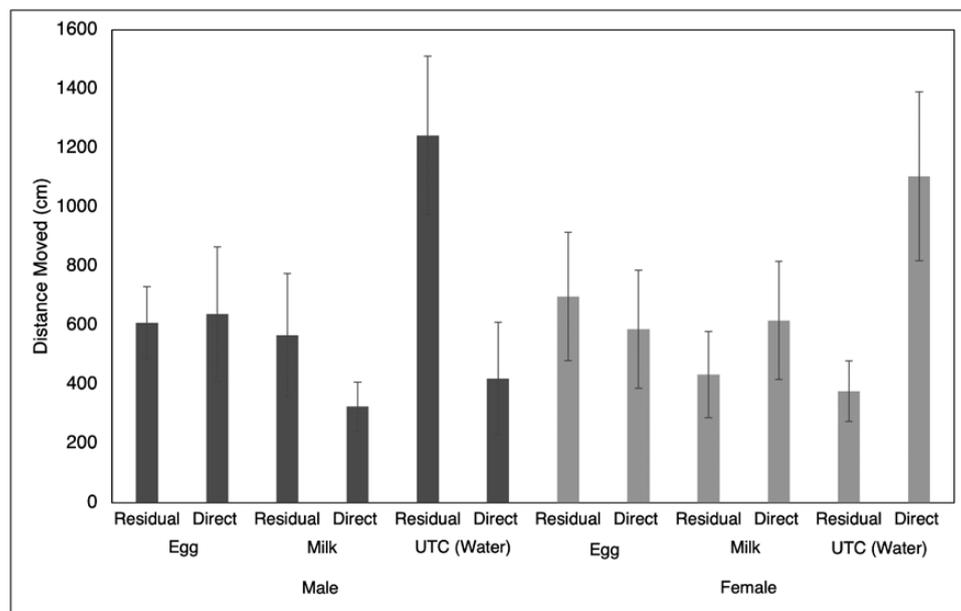


Fig. 3. *Conotrachelus nenuphar* adult speed in cm per second. Insects divided by sex, protein type, and application method. No individual factors significant.

application. Both proteins demonstrated efficacy consistent with data for other insects, which has found that milk and egg are both superior marking materials to soy and wheat, with egg being the most persistent (Jones et al. 2006, Jones et al. 2011). As such, a 7-day collection period is an appropriate period to sample for marked *C. nenuphar* adults as part of a mark–recapture study, although it is possible that field conditions will alter the protein retention. Additionally, movement (as defined by speed, total distance, and height climbed) that are behaviors important for dispersal were unaffected by protein marker type or application method, but females climbed higher than males overall.

Some methods used for mark–recapture studies have been observed to alter the insect’s behavior in ways that make reliable data collection difficult. Use of methods such as mutilations or

physical tags are limited by insect size and may affect behavior once applied. Color markings such as dyes or inks similarly may affect movement, or may trigger cleaning behaviors which reduce movement overall (Garcia-Salaza and Landis 1997). In some cases, internal dyes induce mortality, or do not remain intact for long enough in the insect gut to provide effective marking (Gast and Landin 1966). Colored powders have the benefit of being highly visible on the insect’s body and can glow under a blacklight. However, they may impact the movement of smaller insects, or have retention and survivability issues on very small insects with smooth exoskeletons (Meyerdirk et al. 1979). Too much powder can cause sensory problems and other behavioral issues as well (Cook and Hain 1992). Methods such as radioactive

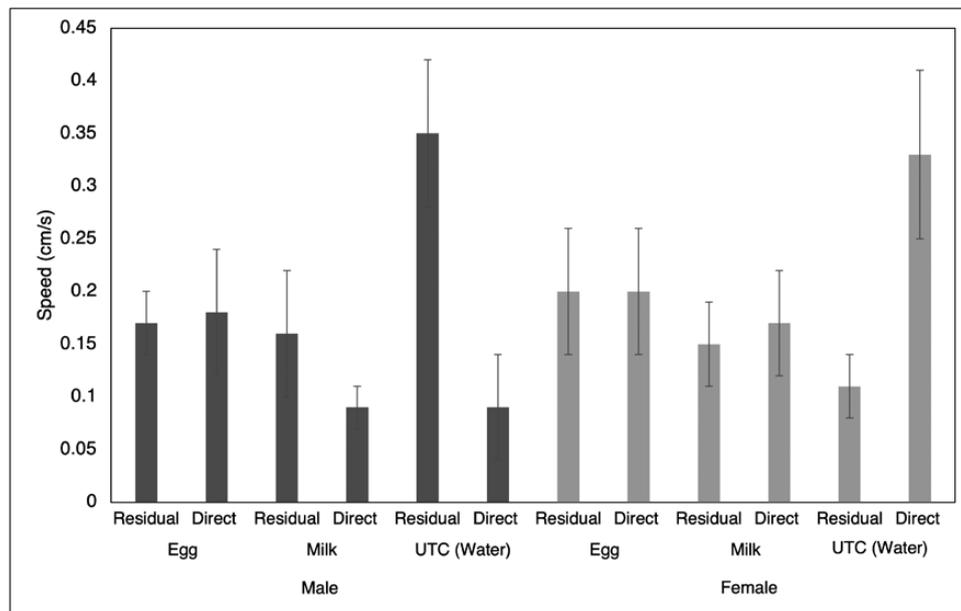


Fig. 4. *Conotrachelus nenuphar* adult total distance moved in 60 min. Insects divided by sex, protein type, and application method. No individual factors significant

Table 1. Optical density scores, days 0–7 post application

Protein	Days since	Direct OD	Residue OD
Egg	0	0.124 ± 0.012	0.124 ± 0.008
	1	0.118 ± 0.019	0.122 ± 0.009
	3	0.124 ± 0.028	0.123 ± 0.016
	5	0.114 ± 0.021	0.119 ± 0.023
	7	0.115 ± 0.006	0.115 ± 0.019
Milk	0	0.102 ± 0.277	0.089 ± 0.016
	1	0.099 ± 0.208	0.124 ± 0.030
	3	0.105 ± 0.019	0.123 ± 0.021
	5	0.089 ± 0.021	0.117 ± 0.032
	7	0.084 ± 0.024	0.104 ± 0.042
Control	NA	0.062 ± 0.0004	

isotope marking have fallen out of favor due to environmental concerns coupled with safer and more inexpensive methods becoming available. Additionally, rare or trace elements used to mark insects were reported to cause issues with development and fecundity in marked insects (Hagler and Jackson 2001).

Methods such as harmonic radar have been tested with *C. nenuphar*, with mixed results. Heavier tags had no significant effect, but lighter tags significantly reduced lateral movement speed (Boiteau et al. 2010). The radioisotope ^{65}Zn has been tested as a marker and *C. nenuphar* immersed in water containing the isotope experienced increased mortality that closely followed the increase in radioactivity. Methods using a protective coat of paint and washing of excess ^{65}Zn from adults resulted in nonsignificant mortality (Lafleur et al. 1985). Enamel paint is an effective method used for determining host preference of adult weevils without behavioral or mortality impacts (Leskey and Wright 2007), however, this method requires mass-rearing of insects and is labor-intensive. Our study shows that *C. nenuphar* marked with these materials can be considered as behaviorally representative when compared with untreated insects.

Use of animal-based protein methods have been used in marking studies on a diversity of insect taxa. Wood-boring

beetles like the emerald ash borer self-mark when emerging from protein-treated trees, allowing researchers to observe their movement without direct observation (Gula et al. 2020). They have also been used to track trophic interactions between predator and prey (Kelly et al. 2012), or between ectoparasite and host (Sivakoff et al. 2016). Using even one protein can help when studying population density and spatial relationships between insect pests on wild and domestic hosts (Hagler et al. 2011, Klick et al. 2016). Additionally, dry protein powders can be used to self-mark insects, which then spread proteins to flowers they forage on. Flowers can then be sampled to determine the foraging range of insects (Hagler et al. 2011).

All the studies mentioned rely on the two assumptions that 1) the marker will last throughout the study period, and 2) that it will not adversely affect their behavior. Overall, our data are consistent with the results of these experiments regarding retention time of the marker, but these studies did not test for behavioral differences between marked and unmarked individuals. Our chosen behavioral measurements of speed, climbing height, and total distance moved laterally are useful proxies for movement behavior when comparing effects of marking materials, or comparing marked and unmarked insects (Kirkpatrick et al. 2020). Additionally, these are important measurements for *C. nenuphar* in particular, as a large share of their dispersal is achieved by walking from tree to tree, then climbing the trunk of a new host. As such, measuring their walking and climbing ability directly correlates to their ability to disperse in the field.

Our data suggest the use of animal protein immunomarkers should be considered a valuable technique in behavioral studies of *C. nenuphar*, with additional implications for testing the behavioral impact of protein marking material on other species.

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Author Contributions

T.L.: Conceptualization, Investigation, Writing- original draft, review & editing, Visualization, Data Curation, Methodology. A.L.N.: Supervision, Writing- review & editing, Methodology, Funding acquisition, Validation. T.C.L.: Supervision, Writing- review & editing, Methodology. A.A.-D.: Supervision, Writing- review & editing, Methodology, Software, Investigation

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