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Short Communication

Marking *Drosophila suzukii* (Diptera: Drosophilidae) With Rubidium or ^{15}N

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ABSTRACT *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) has caused significant economic damage to berry and stone fruit production regions. Markers that are systemic in plants and easily transferred to target organisms are needed to track *D. suzukii* exploitation of host resources and trophic interactions. High and low concentrations of the trace element, rubidium (Rb), and the stable isotope, ^{15}N, were tested to mark *D. suzukii* larvae feeding on fruits of enriched strawberry plants grown in containers under greenhouse conditions. Fly marker content and proportion of flies marked 1, 7, and 14 d after emergence from enriched fruits and fly dry mass were analyzed. Nearly 100% of the flies analyzed 14 d after emerging from ^{15}N-enriched plants were marked, whereas only 30–75% and 0–3% were marked 14 d after emerging from high and low Rb concentration plants, respectively. Rapid Rb decay, strong ^{15}N persistence, and the economics of using these markers in the field to elucidate *D. suzukii* pest ecology are discussed.

KEY WORDS rubidium, ^{15}N, insect marking, strawberry, spotted wing drosophila

Drosophila suzukii Matsumura (Diptera: Drosophilidae) larvae feed on the interior of ripening berry and stone fruits, causing significant economic losses in production regions of Europe and the Americas (Walsh et al. 2011, Cini et al. 2012, Deprá et al. 2014). Insect marking tools can be used to understand movement and dispersal of pests and trophic interactions (Hagler and Jackson 2001), which may lead to improved integrated pest management (Dorn et al. 1999, Jeger 1999, Carrière et al. 2006, Klick et al. 2015). An ideal marker for field experiments is inexpensive, environmentally friendly, easily applied, acquired and persistent, and clearly identifiable (Hagler and Jackson 2001). Protein marking is one such tool and has been successfully used to mark *D. suzukii* in the laboratory (Klick et al. 2014a) and resident flies in a mark–capture study in the field (Klick et al. 2014b). Traceable proteins cannot be incorporated into plants. Systemic marking tools are required to determine what plant hosts are being used by resident flies in nature and to understand particular aspects of pest ecology. The trace element, rubidium (Rb), and the stable isotope, ^{15}N, have been successfully used to mark resident arthropods that have fed on enriched hosts in the field (Stimmann 1974, Nowatzki et al. 2003, Hood-Nowotny and Knols 2007, Tillman et al. 2007). Natural background levels of Rb and ^{15}N are low. The marks are absorbed by small insects and nontoxic to the environment (Berry et al. 1972, Steffan et al. 2001). Our objective was to compare the suitability of Rb and ^{15}N to help determine trophic interactions and sink–source relationships in complex and simple landscapes. We address this by determining 1) the persistence of these markers and percentage marked in adult *D. suzukii* that emerge from marked fruits of treated strawberry plants and 2) the effect of these markers on fly mass (as an indicator of fitness).

Materials and Methods

Marking *D. suzukii*. On 25 May 2012, twenty-five strawberry (*Fragaria* x *ananassa* 'Totem') plants, with green fruits, were transplanted from the field (Oregon State University Botany Farm, Corvallis, OR) to 4.1-liter containers (21.6 by 17.1 cm^2, Anderson Die & Manufacturing, Portland, OR), arranged in a randomized block design, and grown under greenhouse conditions (a photoperiod of 16:8 [L:D] h at 13–24°C). To prevent feral *D. suzukii* from infesting fruits, fine-mesh netting (No-See-Um, Eastex Products, Holbrook, MA) bags were sealed over each plant before fruit turned color. The soil of each plant was drenched with a 250-ml solution five times over a 2-wk period (approximately every 3 d) for each of the following treatments: deionized water (control), 210 ppm N from ^{15}N-enriched NH_4NO_3 (5 atom%, Isotec Inc., Miamisburg, OH), 500, and 5,000 ppm RbCl (99.8% trace metals basis, Sigma-Aldrich, St. Louis, MO; similar

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concentrations as Van Steenwyk et al. 1992, Hood-Nowotny and Knols 2007). Treatments were replicated four times. The water treatment was replicated eight times to provide a set of controls ($n = 4$) in Rb and ^{15}N analysis. Henceforth, treatments will be referred to as control, ^{15}N, 500, and 5,000 ppm Rb.

On 18 June, when ripe fruit was present, each plant was moved from the greenhouse into a transparent cage (22 by 22 by 27 cm^3) topped with fine-mesh netting. Five mature (3–15 d old) laboratory-reared adult *D. suzukii* (as described in Bruck et al. 2011) of each gender were added to each cage and placed in an incubator room at 22°C, 35% relative humidity, and a photoperiod of 16:8 (L:D) h in a randomized block design. Flies were removed after 70 h of exposure. All leaves were removed, and fine-grain white sand (0.15 mm, Unimin, New Canaan, CT) was added to the soil surface to facilitate detection of emerged *D. suzukii*. The first emergence of adults occurred 7 d after the start of fly exposure to fruit; 8–17 d after start of exposure, a mean of 27 (±9 SD) adult flies per plant ($N = 20$) emerged and were collected in respective treatment tubes (32-121BF, Genesee Scientific, San Diego, CA) with 2 cm^3 of Drosophila cornmeal diet (Drosophila Stock Center, San Diego, CA; Markow and O'Grady 2006), which was refreshed every 3 d. A mean subset of 18 (±10 SD) adult flies were harvested from each replicate at 1, 7, and 14 d postemergence, killed by freezing, and stored individually in 1.5-ml microcentrifuge tubes at −80°C. Each sample was dried at 60°C for 48 h and weighed on a microscale (C-35, Orion Cahn, Thermo Electron Corporation, Waltham, MA) prior to mark analysis.

Analysis of Rb-Marked *D. suzukii*. Each fly sample was digested in 6 ml of concentrated HNO_3 using a microwave (Multiwave 3000, Anton Paar) for 40 min (20 min microwave and 20 min cool down) at 140–190°C and 20 bar pressure. Water (18.2 M-Ohm) was used to quantitatively transfer to conical centrifuge tubes with a final volume of 50 ml and HNO_3 concentration of 15% (vol/vol). Rb measurements were made by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a Thermo Scientific X-Series II. Instrument drift was monitored and corrected with an indium (In) internal standard added to every solution to yield a final concentration of 1 ppb In. For the samples, 3 ml aliquots of digested sample were pipetted into 14-ml tubes spiked with In and diluted to 5 ml with 1% (vol/vol) HNO_3. Rb standards were diluted from a commercial standard (Sigma-Aldrich) to make a working range of 0.02–20 ppb Rb. The standards also contained 1 ppb In and 5% HNO_3. The higher acid concentration in the standards was necessary for proper uptake and washout of Rb (Rb in a pure solution of 1% HNO_3 had delayed uptake and washout). Any suppression from the higher acid concentration was corrected by the In internal standard. Apple leaves (SRM 1515, National Institute of Standards and Technology) containing a known amount of Rb were analyzed to verify the analytical accuracy. Fourteen apple leaf samples were analyzed. Rb recovery was very good and ranged from 91–115%. Results from the ICP-MS for the fly samples were measured as ppb Rb and calculated to μg Rb per gram of *D. suzukii*.

Analysis of ^{15}N-Marked *D. suzukii*. Individually dried whole flies were encased in 8- by 5-mm tin capsules (D1006, Elemental Microanalysis Ltd, Okehampton, United Kingdom) and processed by the Oregon State University Stable Isotope Research Unit using an elemental analyzer and Isotope Ratio Mass Spectrometer.

Data Analysis. Statistical analyses were made in R v3.0.3 (R Core Team 2013) with an alpha level of 0.05, or a Bonferroni-adjusted alpha level of 0.006 and 0.017 for analyses involving nine (Rb marker content) and three (Rb effect on fly dry mass) comparisons, respectively (Ramsey and Schafer 2002). Linear mixed-effects models fit by restricted maximum likelihood were used to estimate differences between treatments in marker content and dry mass within each gender (Crawley 2013). Analyses were performed separately for each gender because persistence and marker content varied in male and female flies. The marker content model included treatment by date and treatment by age as fixed effect and fly tube nested within date and nested within container as random effect. Age was included as interaction because the effect of treatment on marker content was expected to change with age. Response variables were natural-log transformed if assumptions of normality and equal variance were not met. The effect of treatment varied by age and by date; therefore, vectors of linear combinations of model coefficients were created to estimate differences in means between treatments. Estimates and confidence intervals were back-transformed, where applicable, and reported with nontransformed means and standard deviations. An individual fly sample was scored as marked if the marker value exceeded the mean value of the control flies plus three times the standard deviation of controls (Stimmann 1974). Cochran–Mantel–Haenszel chi-squared tests were used to determine if marked: nonmarked flies exceeded null expected ratios while accounting for emergence date (Ramsey and Schafer 2002). The dry mass model included treatment by date of emergence as fixed effect with fly age as covariate and fly tube nested within date of emergence as random effect. A drop in deviance test was performed to fit the most appropriate model (Ramsey and Schafer 2002).

Results

Both marker types were detected in flies that emerged from fruits of enriched strawberry plants. Rb content in emerged flies decreased rapidly, whereas ^{15}N persisted in nearly all flies for the entire trial period (14 d; Tables 1 and 2). All (38) 1-d-old flies that emerged from fruits with 500 and 5,000 ppm Rb were considered marked and had more Rb than controls (Tables 1 and 2). Only flies from the high concentration (5,000 ppm Rb) had more Rb and a greater marked: nonmarked ratio than controls throughout the entire trial period (14 d; Tables 1 and 2). The percentage of females marked with Rb 14 d after emerging from the

Table 1. Mean μg Rb/g *D. suzukii* (Rb marking) and ^{15}N atom% *D. suzukii* (^{15}N marking; ± SD), ratio of marked: nonmarked, and percent marked (%) in female and male *D. suzukii* 1, 7, and 14 d after emergence from fruits of strawberry plants enriched with Rb or ^{15}N

Treatment	Mean marker content (± SD)[a]			Marked: nonmarked[b]			Percent marked (%)		
	No. d after emergence			No. d after emergence			No. d after emergence		
	1	7	14	1	7	14	1	7	14
Rb marking									
Female									
Control	15.31 ± 10.96b	14.27 ± 5.21b	8.56 ± 9.16b	1: 8b	0:22b	1: 25b	11	0	4
500 ppm Rb	167.23 ± 123.90a	13.70 ± 13.02a	14.65 ± 9.03b	11: 0a	1:22b	1: 31b	100	4	3
5,000 ppm Rb	608.66 ± 126.37a	66.96 ± 44.94a	75.83 ± 49.99a	7: 0a	5:2a	21: 7a	100	71	75
Male									
Control	14.41 ± 5.23c	17.06 ± 5.55b	8.04 ± 7.83b	0: 7b	0:13b	0: 30b	0	0	0
500 ppm Rb	136.76 ± 55.13b	19.79 ± 13.92b	15.38 ± 8.60b	12: 0a	1:12a	0: 20b	100	8	0
5,000 ppm Rb	1,080.64 ± 998.35a	178.63 ± 393.87a	32.24 ± 17.85a	8: 0a	7:4a	13: 31a	100	64	30
^{15}N marking									
Female									
Control	0.37 ± 0.0008b	0.37 ± 0.0007b	0.37 ± 0.0007b	0: 7	0: 30	0: 26	0	0	0
^{15}N	0.40 ± 0.0144a	0.39 ± 0.0109a	0.39 ± 0.0089a	10: 1	20: 0	28: 0	91	100	100
Male									
Control	0.37 ± 0.0005b	0.37 ± 0.0006b	0.37 ± 0.0006b	0: 5	0: 19	0: 20	0	0	0
^{15}N	0.41 ± 0.0032a	0.40 ± 0.0095a	0.40 ± 0.0107a	6: 0	28: 0	31: 0	100	100	100

[a]Different letters within the same gender of a marker and same column are significantly different using linear mixed-effects model. Statistical output is presented in Table 2.

[b]Different letters within the same gender of a marker and same column are significantly different in number of individuals marked: nonmarked based on Cochran–Mantel–Haenszel chi-squared test. Statistical output is presented in Table 2. Because no flies were considered marked in the control and nearly 100% marked in the ^{15}N treatment, no statistical comparisons were possible (i.e., the odds ratio could not be calculated).

Table 2. Output of statistical comparisons for Rb marker content and ratio of marked: nonmarked 1, 7, and 14 d after emergence from fruits of strawberry plants enriched with Rb.

Treatment comparison	df[a]	1 d after emergence			7 d after emergence			14 d after emergence		
		Estimate	95% CI	*P*	Estimate	95% CI	*P*	Estimate	95% CI	*P*
Rb marker content[b]										
Female										
500—control	37	15.08	3.02–75.27	<0.0001**	8.43	1.60–44.51	0.0006*	2.40	0.82–7.03	0.0230NS
5,000—control	37	72.07	13.09–396.74	<0.0001**	10.64	2.22–51.12	0.0001*	10.01	3.66–27.34	<0.0001**
5,000—500	37	4.78	0.89–25.62	0.0100NS	1.26	0.27–5.94	0.6629NS	4.17	1.39–12.54	0.0005*
Male										
500—control	36	8.47	1.73–41.36	0.0004*	3.03	0.25–36.67	0.2025NS	1.09	0.12–9.52	0.9201NS
5,000—control	36	149.15	27.34–813.58	<0.0001**	46.45	4.00–539.96	<0.0001**	29.53	2.86–305.30	0.0002*
5,000—500	36	17.62	3.04–102.20	<0.0001**	15.33	1.22–192.25	0.0033*	27.38	2.67–280.70	0.0002*
Rb marked: nonmarked[c]										
Female			χ^2			χ^2			χ^2	
500—control	1		11.67	0.0006*		0.83	0.3613NS		0.30	0.5827NS
5,000—control	1		8.13	0.0044*		9.16	0.0025*		19.59	<0.0001**
5,000—500	1		Na	Na		7.51	0.0062*		21.30	<0.0001**
Male										
500—control	1		12.35	0.0004*		0.88	0.3496NS		Na	Na
5,000—control	1		8.77	0.0031*		6.34	0.0118*		8.21	0.0042*
5,000—500	1		Na	Na		3.40	0.0654NS		5.63	0.0177*

Estimates represent a multiplicative increase in median marker content in the 500 or 5,000 ppm Rb treatments.
Back-transformed estimates and confidence intervals (CI); NS, nonsignificant; *, $P < 0.006$; **, $P < 0.0001$.

[a]One less df in males because males were missing from one of the emergence dates.

[b]Linear mixed-effects model with a Bonferroni-adjusted alpha level of 0.006 for nine comparisons.

[c]Cochran–Mantel–Haenszel chi-squared test with an alpha level of 0.05.

high concentration fruits was 75% or 2.5 times that of males from the same treatment (Table 1). Detailed statistical outputs of treatment comparisons are presented in Table 2.

All flies (except 1 out of 124) analyzed for ^{15}N were considered marked after emergence from enriched fruits at all time periods tested (Table 1). Female and male flies that emerged from fruits treated with ^{15}N were estimated to have 7 ($P < 0.0001$, CI = 1.06, 1.08) and 8% ($P < 0.0001$, CI = 1.07, 1.09) more ^{15}N atom% per *D. suzukii* than controls, respectively, regardless of age. Because no control and nearly 100% of ^{15}N flies were considered marked at all time periods, no statistical comparisons were possible (i.e., the odds ratio could not be calculated; Table 1). The treatments had no effect on female and male dry mass.

Discussion

The ^{15}N mark was highly persistent in flies that emerged from enriched plants, but Rb rapidly decayed with time in flies from the low concentration and slightly less so in flies from the high concentration treatments. In other studies, rapid loss of Rb occurred 2–4 d after lygus and western corn rootworm stopped feeding on enriched plants (Fleischer et al. 1986, Nowatzki et al. 2003), but persisted for up to 27 d in Medfly (Van Steenwyk et al. 1992). Food source may affect Rb decay as the rate of Rb loss correlated with KCl in food of *Drosophila* spp. and *Megaselia scalaris* (Fairbanks and Burch 1968). Furthermore, KCl is physiologically similar to the RbCl used in this study and was abundant in excretions of insects (Ramsay 1953).

Similar to our findings, all treated aphids that fed on ^{15}N-enriched oat plants remained marked 10–16 d after transfer to nonenriched oats (Nienstedt and Poehling 2000). Several factors may explain why ^{15}N was persistent in flies: nitrogen is retained from food as proteins to make tissue and muscle in immature arthropods (Speight et al. 2009), nitrogen can be resorbed (Mira 2000), and insects typically retain signatures of larval foods as adults (Hood-Nowotny and Knols 2007).

The markers tested at given concentrations did not affect fly dry mass. However, similar Rb concentrations tested in other studies have been shown to have adverse effects on insects. High concentrations of Rb (6,000 ppm to diet) affected development, fecundity, and longevity of tufted apple bud moth (Knight et al. 1989), and concentrations above 5,000 ppm reduced survivorship in blueberry leaftier (Polavarapu et al. 1992). *D. suzukii* had a similar amount of Rb as Medfly (Van Steenwyk et al. 1992). Spraying or injecting 10,000 µg Rb/ml on coffee fruit resulted in Medflies with 680–1220 µg Rb/g, which did not have any adverse biological effects. Adverse effects of ^{15}N have not been reported.

Costs of *D. suzukii* analysis for Rb and ^{15}N were seven- and eightfold higher, respectively, than egg protein marking (Klick et al. 2014a,b). However, ^{15}N and Rb may be used to answer other research questions, such as the origin of a fly and trophic interactions, but may be limited in scale by the high costs. Field marking material costs may be offset if the rate of decay of ^{15}N is shown to be much slower than egg protein. Rb was less favorable than ^{15}N as a marker because of shorter persistence and the risk of negative effects on insect biology. Future work should determine the role of other host material on marker effect and the persistence of ^{15}N in *D. suzukii*. The ^{15}N mark successfully labeled *D. suzukii* via enriched plants and was highly persistent without any known adverse biological effects; hence, providing a tool for tracing pest exploitation of host resources and other trophic interactions.

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