Use of a Highly Sensitive Immunomarking System to Characterize Face Fly (Diptera: Muscidae) Dispersal From Cow Pats

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Environ. Entomol. 43(1): 116–122 (2014); DOI: http://dx.doi.org/10.1603/EN13139

ABSTRACT We tested an immunomarking system that used egg white as marker and enzyme-linked immunosorbent assay as a detection assay to characterize face fly (*Musca autumnalis* DeGeer) dispersal from cow pats in a pastured beef cattle operation. In microcage assays, adult flies acquired marker after contact with cow pats that were treated with marker and field aged up to 11 d. In arena assays on sprayed full-size cow pats, 77% of eclosed face flies acquired the marker. In a field-marking study, four applications of egg white marker were applied on freshly deposited cow pats over a summer at two peripheral paddocks to a main grazing pasture of ≈50 head of beef cattle. Of the 663 face flies captured, 108 were positive for the egg white marker (16.3%). Of the marked flies, ≈ twofold more male than female flies were captured. Sex-specific dispersal distances were roughly equal up to 450 m, with 11% of female flies dispersing >450 m. Dispersal capability of face flies is discussed in relation to efficacy of rotational grazing and other IPM strategies.

KEY WORDS *Musca autumnalis*, insect dispersal, mark–recapture

Dispersal of insects has been studied in many systems using a variety of mark–recapture methods (Hagler and Jackson 2001). Insects are usually marked and released from one or more sites, and traps are used to map their movement patterns. Previous mark–release–recapture work with insects of medical and veterinary concern has almost exclusively used fluorescent powders (Reisen and Lothrop 1995, Walton et al. 1999, La Corte Dos Santos et al. 2004, Maciel-de-Freitas et al. 2007, Casanova et al. 2009). However, the development of immunomarking procedures offers a potential new method for studies in medical and veterinary environments (Jones et al. 2006, Hagler and Jones 2010, Jones et al. 2011). These new markers are cost-effective, highly sensitive, well suited to mark–capture studies, and improve information on the potential impact of insect pest dispersal (Cameron et al. 2009). Furthermore, they can be scaled to study dispersal over short (Horton et al. 2009) and long distances (Boina et al. 2009) .

Face flies, *Musca autmnalis* De Geer (Diptera: Muscidae), have a significant effect on livestock and dairy production throughout North America (Moon 2002). Face flies may impact cattle behavior and productivity of beef cattle operations and can vector the causative agents of various cattle diseases such as pink eye (Hall 1984), bovine thelaziasis (O'Hara and Kennedy 1991), hemorrhagic bovine filariasis (Bech-Nielsen et al. 1982), and brucellosis (Cheville et al. 1989). Control methods have focused on adulticides, traps, and larvicides. However, efficacy of insecticide applications is situational, and more research is needed to examine the reasons behind this variability. Larval face flies inhabit freshly deposited cow pats, and fourth-instar larvae exit the pat to pupate nearby (Krafsur and Moon 1997). We exploited this series of life history traits in laboratory and field experiments by applying an immunomarker directly to the surface of cow pats.

Face flies are capable of dispersing 30–300 km in a single season, as observed during the initial years after their U.S. introduction (Sabrosky 1961). Moon and Krafsur (1995) conducted a mark–release–recapture study with face flies using fluorescent dust. However, it focused on fly pterin content to calibrate age-grading models, and did not explicitly measure dispersal distances. Dispersal ability directly impacts fly ability to locate resources and reflects the propensity for emerging face fly adults to seek the nearest herd of cattle using olfactory and visual cues. Rotational grazing (RG) continues to be promoted as a viable grazing strategy for beef cattle production (Briske et al. 2008), with purported benefits of increased production (animal and plant). The benefits of RG may be diminished if cattle pest insects are able to disperse great distances from their natal habitats in search of suitable hosts.

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Herein we report on the use of egg white as an immunological marker to track adult face fly dispersal from cow pats.

Materials and Methods

Fly Colony Maintenance. Face flies were obtained from the Kansas State University Entomology Department susceptible laboratory colony, initially collected from northeastern Oklahoma in 1978 (Broce and Elzinga 1984). Face fly larvae were reared on freshly thawed manure using standard methods (Moon 1980). Adults were given water on paper wicks, table sugar, and cow liver ad libitum. Oviposition was into freshly thawed cow manure. Adult face flies (control samples) were reared in Bug Dorm-1 Insect Rearing Cages (30 by 30 by 30 cm, Product #1452, BioQuip Products, Rancho Dominguez, CA) in an egg-free rearing room with a photoperiod of 16:8 (L:D) h, temperature ranging from 20 to 25°C, and relative humidity ranging from 45 to 55%.

Laboratory Studies. Our experimental herd grazed a fenced irrigated sage scrub or grassland area of ≈ 20 ha, 4 km northeast of Prosser, WA (46° 12′24″ N, 119° 46′8″ E), supplemented with alfalfa hay. The experimental herd consisted of mixed age (<1–13 yr) Herefords (30%), Angus (25%), and Angus–Hereford (Black Baldy; 45%), totaling 50 head. We tested the ability of face flies to pick up the egg white marker by allowing them to walk on the marker-sprayed crust of aged cow pats. Freshly deposited dung (<5 min) was collected from the experimental herd and transported in plastic bags to a fenced pasture within the experimental site that excluded all cattle. The pasture was irrigated by seepage and consisted of a mixture of green forbs suitable for grazing. We placed five 3.5-liter cow pats within this pasture in a row 1 m apart and allowed them to dry for 1 h. Egg white marker was mixed with a 0.3 g/liter sodium (tetra) ethylenediamine tetra acetate (EDTA; S657; Sigma-Aldrich, St. Louis, MO) and tap water to make a 10% marker solution. Cow pats were sprayed with marker solution until the point of dripping. After 24 h, a crust developed on the artificial pats, and samples of this crust (1 cm in diameter by 3 mm in depth) were taken with a clean spatula and placed in labeled microcentrifuge tubes. The spatula was rinsed with 70% isopropyl alcohol and wiped with a clean Kimwipe between crust samples.

Crust samples were taken at 1–5, 7, 9, 11, 14, 18, 22, and 30 d postspraying and stored at −20°C. Newly eclosed face flies (≤1 d old) were anesthetized with CO_2 and put into horizontally oriented 1.5-ml microcentrifuge tubes (n = 5 tubes per date; #022363212, Eppendorf, Hauppauge, NY) with a 15 mg subsample of sprayed pat crust that was cut to fit precisely into the tube, creating a uniform surface that ensured flies contacted only the field-aged part of the crust. Control face flies were placed in tubes with untreated pat crust. Flies revived quickly and were allowed to walk over the crust for 1 h. Tubes with flies were placed in a −20°C freezer to immobilize but not kill (20 min). After immobilization, flies were removed from tubes with a clean toothpick and placed in a microcentrifuge tube with 1 ml tris-buffered saline (TBS, pH 8.0; T-664; Sigma-Aldrich) and left to soak 3 min. Flies and toothpick were removed and discarded. Tubes with 1 ml TBS were held at −20°C until analysis by enzyme-linked immunosorbent assay (ELISA) as described in the Immunoassays section. Rainfall for the entire sampling period (5 August to 5 September 2008) was 5.1 mm (WSU Hamilton Station; http://weather.wsu.edu/awn.php).

To test the ability of face flies to acquire the egg white marker as they emerged from pupae near marked cow pats, we conducted arena experiments. Arenas consisted of five plastic tubs (36 by 36 by 18 cm with lids) with four 8-cm-diameter ventilation holes on sides of tub covered with fiberglass window screen (screen hole size 1.5 mm on side). A 2-cm layer of clean playground sand (Quikrete Play Sand, Home Depot, Atlanta, GA) was placed into each tub, and aged cow pats (≈7–10 d old from the experimental herd, ≈30 cm in diameter) were placed on the sand. The pat and sand of each tub were sprayed with egg marker solution (same as above) until small droplets on pat began to drip. The pat was placed outdoors in full sun, and the egg marker solution was allowed to dry on the pat for 1 h. Fifty face fly pupae (Kansas strain reared as described above, 5 d since pupation) were placed on the sand around the edge of the pat. To catch flies after they emerged, picked up marker, and began to fly, we used Delta Trap white sticky liners (Scentry Biologicals, Billings, MT) glued to the underside of tub lids. Using a clean putty knife, we scraped ≈80% of sticky material off liners before deployment, as an overabundance of sticky material covers insects and inhibits release of marker when insects are soaked in TBS. Lids with liners were placed on tubs and set in the face fly-rearing room described previously. Tubs were monitored for emergence of face flies. Forty-eight hours postspraying, tubs were placed in a −20°C freezer for 1 h to kill face flies that did not land on sticky cards. Face flies that had adhered to the sticky traps were removed with a clean toothpick and placed in microcentrifuge tubes filled with 1 ml of TBS for 3 min. Face flies were then removed with their toothpick and discarded and the buffer frozen at −20°C until ELISA analysis. Toothpick "swabs" of the cards served as contamination controls in ELISA. Flies killed by freezing but not on sticky cards were collected from tubs with cleaned tweezers, placed in 1.0 ml TBS, and treated as above.

Field Study. A field study was conducted during the summer of 2009 in and around a 20-ha pastured beef cattle operation in Prosser, WA (experimental herd described above; 46.22° N, 119.72° W). Livestock operations bordering our trapping area included an irrigated dairy cow pasture (≈100 head of Holsteins) to the immediate east, two angus herds (≈20 head) to the southeast, and small farms (<5 cows) to the north, west, and south. To recover marked face flies, we used white Delta Traps with white sticky liners (scraped as above) attached to fencing 1 m above the ground.

Sixty Delta Traps were laid out in 20-m intervals in transect lines in the four cardinal directions (N–S–E–W) originating from the center of the two spraying sites of 0.9 ha, and each trap transect line was 300 m long. Thus, the maximum possible detectable distance traveled by any marked fly from its point of marking origin in the sprayed site was ≈600 m (two transect lengths N–S). The first spray site was named the "Little Pasture" (hereafter LP), and is an irrigated paddock located in the southeast corner of the main grazing pasture; this site was sprayed on 25 August and 23 September. The second spray site was named the "Northeast Pasture" (hereafter NEP), and is an irrigated paddock located in the northeast corner of the main grazing pasture; this site was sprayed on 10 August and 8 September. The 2-wk interval between sprayings allowed marked flies from a previous spraying to die, as their average adult life span is reported to be ≤11 d (Krafsur and Moon 1997), and also allowed for marker degradation from previous spraying events at a given site. To aggregate pats and properly age them, our herd was confined with permanent fence (LP) or encouraged to aggregate with alfalfa hay supplementation twice daily (NEP) for 48 h in spraying areas 9 d before marker application. The sequence of events for a given location–spray date was as follows: aggregate herd for pat deposition (day 1 to day 2), move herd away (2 d), allow for larval development in cow pats (day 2 to day 10), spray (day 10), set new sticky cards out in Delta Traps (day 11), remove face flies from sticky cards (day 17). We diluted the egg white protein to 10% with tap water mixed with 0.3 g/liter EDTA. Egg marker applications were made using a spray rig attached to an all-terrain vehicle. The spraying rig consisted of a 14-gallon plastic tank (G&R Ag Products, Pasco, WA), a diaphragm pump (model 2087–593-135; SHURflo, Cypress, CA), and a GunJet sprayer (No. 30L, 1.5-mm orifice, 5500 adjustable nozzle; Spraying Systems, Wheaton, IL). The pump capacity was 1.9 liter/s, and our field line pressure was 103 kPa. Individual pats were sprayed until solution just began to puddle (≈3 s). One 14-gallon tank of egg white marker solution was enough to cover ≈ 300 pats. Delta traps (transects for NEP and LP) received fresh sticky liners at day 10 after each spraying. Trapped flies from all transect trap lines were removed from sticky liners at the field site with a clean toothpick, sexed, and placed in a microcentrifuge tube with 1 ml TBS and left to soak 3 min. After soaking, flies were removed with a second clean toothpick and tubes with sample buffer were held at −20°C until analysis with ELISA as described below. Rainfall for the entire sampling period (10 August to 1 October 2009) was 5.3 mm (WSU Hamilton Station; http://weather.wsu.edu/awn.php). To control for egg protein contamination and to check for homologous protein cross reactions, we included toothpick swipes of field-deployed trap liners, swipes of laboratory-clean trap liners, egg-free laboratory reared face flies recovered from trap liners placed in their egg-free rearing cages, and samples of TBS in our immunoassays.

Data Analysis. In all studies, samples were considered marked if the ELISA optical density (OD) readings were 4 SD above the mean of unmarked control insects (=positive threshold; Jones et al. 2006). This is conservative compared with methods that use 3 SD (Crowther 2001) but provides more protection against false positives. The increased false-positive protection is useful because there is greater concern with falsely declaring an unmarked insect to be marked than the converse.

To analyze the relationship between probability of positive mark and days of field exposure to pat crust in marker acquisition assays, we conducted logistic regression analysis using R (R Core Team 2013) to generate maximum likelihood estimates of the logistic model parameters. The Likelihood Ratio Test uses twice the negative log of the likelihood ratio, called the deviance (D; Faraway 2006), to make inferences about how close the fitted values from a smaller (residual) model comes to the perfect fit of the saturated (as many parameters as cases) model. Provided that the dependent variable is truly binomial and the sample size is relatively large ($n > 5$), the deviance is approximately chi-squared distributed ($D \approx \chi^2$), where values of $D < \chi^2_{\text{critical } (\alpha = 0.05)}$ are evidence of adequate fit. Likelihood Ratio Tests (G-Test; Sokal and Rohlf 1995) were performed on frequencies of face flies captured and sorted by sex and marker status. The null hypothesis being that equal numbers of male and female flies would be trapped and that of the flies trapped, equal numbers of male and females would be positive for mark.

Immunoassays. We performed protein-specific ELISA using C6534 as the primary antibody for chicken egg albumin (Sigma-Aldrich). The secondary antibody used for immunoassays were donkey anti-rabbit IgG (H+L) with a peroxidase conjugate (31458; Pierce Biotechnology, Rockford, IL). The egg protein (antigen) applied to the cow pats were dilutions of All Whites (Papetti Foods, Elizabeth, NJ). Immunoassays were all performed as indirect ELISAs (Crowther 2001). All incubations, unless otherwise specified, were performed at 37°C on dry block microplate heaters. Clean wooden toothpicks were used to remove individual flies from traps and place them in 1.5-ml microcentrifuge tubes containing 1 ml TBS, to which 0.3 g/liter EDTA was added. Toothpicks were left in the tube to keep flies submerged in TBS for 3 min. The flies were discarded to minimize the amount of extraneous protein removed from their bodies that might bind competitively to the plate and reduce our ability to detect the egg protein. All TBS solutions were prepared in water that was filtered, de-ionized (>18 MΩ cm), and distilled using a Barnstead Fi-streem III glass still (Barstead International, Dubuque, IA). Details of the immunoassay protocol, including microplate incubation, well washing, addition of blocking solution and antibody, and optical density reading are described in Jones et al. (2006). Tubes were frozen at −20°C until thawing for immunoassay.

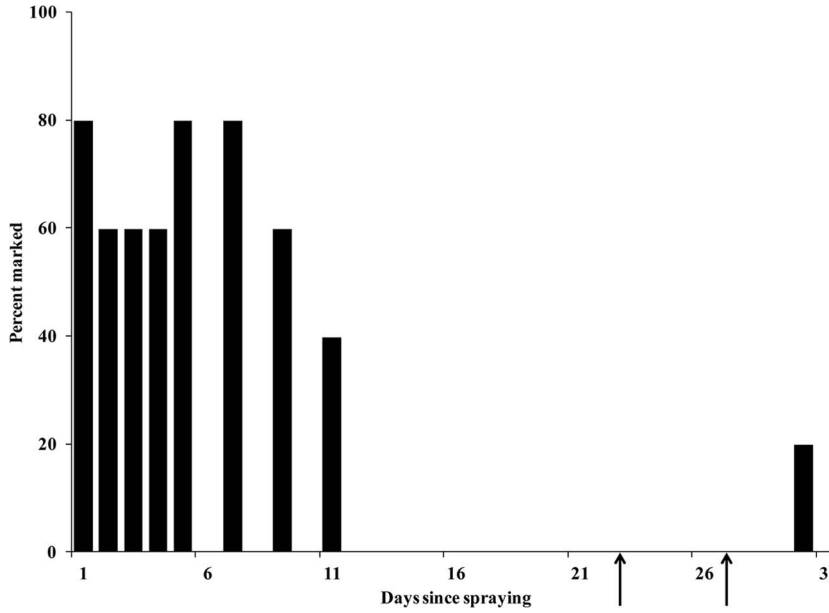

Fig. 1. Percent of face flies positive for mark in "walk on" study of field-sprayed cow pat crust. Walk on assays (1 h) were conducted for crust samples taken 1–5, 7, 9, 11, 14, 18, 22, and 30 d postspray, $n = 5$ for each sampling date. Arrows indicate days of rain (5.1 mm total).

Results

Laboratory Studies. In the marker acquisition study, there was a significant effect of time after cow pat spraying on ability of adult face flies to pick up marker while walking over aged cow pat crust in microcentrifuge tubes. Face flies walking on marked crust had difficulty picking up the marker on crust left in the field longer than 7 d, with no flies picking up sufficient marker for positive ELISA for crust sampled on day 14, 18, and 22 (Fig. 1). The results of logistic regression for the marker acquisition suggest that the fit for the null model (no predictors and just an intercept term) is inadequate ($D = 82.577$; df = 59; $P = 0.023$). However, including the predictive effect of day in the model gives a model with sufficient fit ($D = 65.383$; df = 58; $P = 0.236$). In the arena study, the percentage of face flies emerging in arena replicates ranged from 4 to 58%, with an overall eclosion rate of 27.6%, and an overall positive mark for 78.3% of flies captured in the five arena replicates (Table 1).

Field Study. During the summer of 2009, there were four spraying events (NEP on 10 August and 8 September, and the LP on 25 August and 23 September). Face flies were collected on 18 August (first NEP spray), 29 August (first LP spray), 16 September (second NEP spray), and 1 October (second LP spray). In total, 663 face flies were removed from sticky traps and analyzed for marker; of those, 108 were positive (16.3%, Table 2). There were more males captured than females for marked (65.7%; $G = 10.89$; $df = 1$; $P < 0.001$) and unmarked (63.6%; $G = 41.61$; $df = 1$; $P < 0.001$) flies. A graph of number of marked flies captured as a function of distance from the center of spray areas (Fig. 2) suggests differences in dispersal patterns by sex. For percentages of total marked flies trapped by distance interval, we found 43% of all marked females and 32% of all marked males were recovered from traps ≤100 m from the center of their respective marker spraying sites. For marked flies recovered from traps ≤250 m from spraying sites, 76% were female compared with 75% male. For marked flies recovered from traps ≤450 m from spraying sites, 89% were female, whereas 100% were male. Eleven percent of marked female flies were captured beyond the range of marked male flies. Further, only 4.6% of all marked flies were captured in traps in the 1- to 49-m interval,

Table 1. Arena experiment

Replicate	Percent eclosed (n)	Percent positive (n)
1	4.0 (2)	50.0 (1)
2	32.0 (16)	75.0 (12)
3	22.0 (11)	100.0 (11)
4	22.0 (11)	72.7 (8)
5	58.0 (29)	72.4 (21)
Overall	27.6 (69)	78.3 (54)

Table 2. Summary statistics for 2009 field ELISA trials

Treatment	Portion	Statistic
	Total flies captured	663
	Total males (%)	424 (64.0)
	Total females (%)	239 (36.0)
Marked	Total (%)	108 (16.3)
	Male (%)	71 (65.7)
	Female (%)	37 (34.3)
Unmarked	Total (%)	555 (83.7)
	Male (%)	353 (63.6)
	Female (%)	202 (36.4)

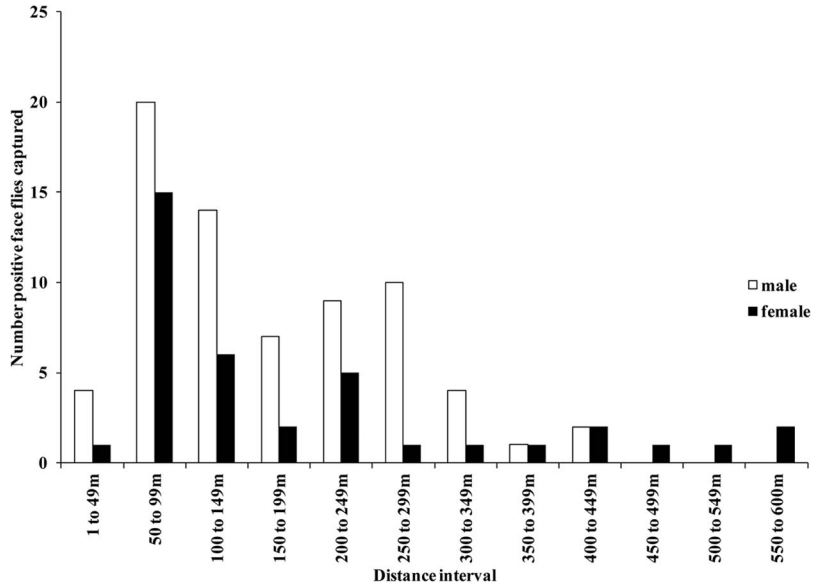

Fig. 2. Number of marked face flies captured by distance interval from spray site.

while 86% of all marked flies were captured in traps >49 m but <350 m from spraying sites (Fig. 2). There was a significant difference ($t = 15.3$; $df = 106$; $P < 0.001$) in mean (1 SE, n) dispersal distance when comparing sex of face flies, with females flying 193.2 m (24.7, 37) and males flying 169.9 m (11.9, 71), indicating a difference of 12.1%, with females flying farther than males by a factor of 1.14.

Discussion

The relatively high percentage of face flies picking up marker from sprayed substrate and the trend for decreasing percentage marker pickup as a function of field-age of substrate is similar that seen in previous studies (Jones et al. 2006, 2011), although the substrate types were different (cow pats vs. apple trees). Our studies showed that to pick up enough marker for a positive ELISA, face flies needed to walk on marked pat crust ≤11 d since spraying. These marker-pick-up results determine the best timing of egg white spray applications for mark–recapture in our field study, and will be of use in similar studies of coprophagous diptera. Because face fly oviposition occurs immediately after pat deposition by cows, and the average developmental time from egg to emerging adult is usually ≈14 d (Krafsur and Moon 1997), the timing of egg white spraying on pats should be on or around 11 d after deposition. Other immunomarking studies have used dry powders of wheat, soy, or powdered milk (Jones et al. 2006), and these dry markers may be less likely to degrade and may leave greater residues on target flies when they visit marked substrates. However, compared with aqueous solutions, dry powders have a greater propensity to become airborne and contaminate and thus confound any inference on the geographic origin of marker pickup. This is especially relevant in experimental areas subject to frequent winds.

Nearly 80% of emerging face flies tested positive for egg white marker in our arena study. Previous "arena" designs had similar rates of marker pickup (Hagler and Jones 2010), although the arena and exposure time was of smaller scale, and the target insects were agricultural pests. The present arena study is roughly comparable with unpublished results focused on hymenopteran parasitoids (see Hagler and Jackson 2001). However, the rate of marker pickup for face flies emerging from a "host" (cow pat) was much larger when compared with hymenopteran parasitoids picking up surface marker as they emerged from treated hosts.

Previous field studies suggest that egg marking rates greater than ours can be achieved (Jones et al. 2006). However, differences in environmental locations (orchards vs. cattle pastures) and target insect life histories (agricultural vs. cattle pests) may impose limits on immunomarking system outputs. Face flies are reported to migrate considerable distances in search of suitable hosts (Krafsur and Moon 1997). This emigration effect from adjacent cattle operations may have diluted our trap catch, and is one explanation for the relatively low percentage of marked flies recovered. Another factor possibly affecting our catch was trap placement. Using the four cardinal directions for trap transect lines left wide gaps for marked flies to move beyond our experimental area. A better design might include concentric trap placement of increasing radii (Schoof et al. 1952, Walton et al. 1999).

Insect movement can be substantial (Jones et al. 2006), especially for agricultural pests that are excellent dispersers (Hagler and Jackson 2001). Filth flies are also excellent dispersers (Drummond et al. 1988). However, when suitable habitat is found, filth flies will

tend to aggregate (Schoof and Siverly 1954). Face flies are attracted to and tend to aggregate around cattle (Krafsur and Moon 1997), and the observed patterns of highest trap catch in the current study (>49 m and <350 m, Fig. 2) were most likely caused by a tendency of the experimental herd to aggregate at those distances from the egg marker spray sites.

Rotational grazing and its effect on plant and animal production has been thoroughly reviewed (Briske et al. 2008), and there is some evidence that rotational grazing may be useful for reducing gut parasites (Larsson et al. 2006), but we know of no studies focused on how rotational grazing may allow cow herds to avoid being discovered by pest flies that emerge from their pats on previously grazed pastures. Although this study did not examine fly loads directly, it does offer evidence that face flies can move great distances away from emergent pastures, nearly 600 m in two cases. This suggests that for rotational grazing regimes to impact the ability of face fly host location, cattle must be rotated many hundreds of meters from their last grazed area. This may not be feasible in many cattle operations.

Although immunomarking has its own unique set of complications when applied to flies emerging from cow pats, it performed remarkably well in this particular beef production system. The low cost, high sensitivity, high rate of egg marker pickup, egg marker resistance to degradation, egg marker resistance to trap adhesive reduction in ELISA efficiency, and ease of mass scale marker application makes this technique a superior alternative to other mark–recapture methods for filth fly dispersal studies (Jones et al. 2011). This immunomarking method should find use in studies of arthropod-borne disease ecology in general and face fly population ecology in particular. One important use of immunomarking data will be estimations of disease transmission efficiency using vectorial capacity (Garret-Jones and Shidrawi 1969). Mark–release–recapture experiments provide the best method of estimating vector population parameters that make up the vectorial capacity equation, such as survivorship, population size, gonotrophic cycle duration, and host-feeding patterns (Casanova et al. 2009). The dispersal information provided by immunomarking studies of vectors, including the pink eye vector in this current study, is essential to understanding the dynamics of vector-borne disease transmission in agricultural and public health systems, and is essential for planning appropriate control measures.

Acknowledgments

We thank the staff of the Environmental and Agricultural Entomology Laboratory for their technical assistance. Tawnee Wilburn-Melton, our immune-chemist at the Insect Ecology and Behavior Laboratory, Tree Fruit Research and Extension Center, WSU-Wenatchee, was instrumental to the success of our study. This work was funded by a USDA Risk Management Assessment (RMA) grant and a USDA Pest Management Alternative Program (PMAP) grant to DB Walsh.

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Received 11 May 2013; accepted 6 December 2013.