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Detection of Fluorescent Powders and Their Effect on Survival and Recapture of *Aedes aegypti* (Diptera: Culicidae)

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Abstract

The use of insect markers, such as fluorescent powders, is a useful tool for studying ecological and epidemiological questions. Evaluating their effect on vectors of human disease agents, such as the invasive mosquito vector *Aedes aegypti* (Linnaeus), is crucial for their practical and reliable use, especially in parameters linked to the risk of disease transmission such as adult survival, dispersal, and host-seeking. Seven fluorescent powders (Hercules Radiant, DayGlo (DG), Risk Reactor (RR), and Angstrom Technologies), applied externally on cohorts of *Ae. aegypti* female mosquitoes, were tested to determine their impact on survival and recapture by baited mosquito traps, and their detectability after being exposed to controlled laboratory and semifield environments. There were no significant differences in survival among marked and unmarked females across all powders. Marked females were significantly less likely to be captured in baited traps relative to unmarked females, except for one of the DG powders. All females remained visibly marked on five parts of their body for 30 d (under both environments), except for one of the RR powders. The tested powders and application method are suitable for tracking mosquitoes throughout most of their lives under different environments, without significantly affecting their survival, but with potential impact on recapture by baited traps, possibly due to effects on senses or other physiological traits.

Key words: marking, fluorescent, powder, mosquitoes, Culicidae, vector

In addition to their nuisance biting, mosquitoes are important vectors of pathogens that cause human and animal diseases and affect not only the health of those who suffer illnesses, but also the development and economies of countries with high disease burdens (Danasekaran et al. 2014). *Aedes aegypti* (Linnaeus) is an important vector of arboviruses that have a severe impact on public health, including dengue, yellow fever, chikungunya, and Zika viruses (Zellweger et al. 2017). Aspects of its biology such as adult survival, density, ethology, demography, population dynamics, dispersal capabilities, trophic-level interactions, and other ecological interactions are key factors that must be studied to establish effective public health policies focused on prevention and vector control and to establish prediction risk transmission models (Verhulst et al. 2013, Alcalay et al. 2018).

Different ecological and experimental approaches have been deployed to elucidate parts of the abovementioned parameters, with marking methods being one of the tools most commonly used in studies. Most of the available insect markers have also been used in mosquito studies, including the use of dyes, paints or inks, powders (also known as 'dusts'), trace elements, radioactive or stable isotope materials, proteins, and phenotypic mutants or genetically modified individuals (Silver 2008, Hagler and Jones 2010, Guerra et al. 2014, Benedict et al. 2018).

Each marking method varies in its efficiency, practical and logistical complexity, applicability, cost, advantages, disadvantages, and impact on marked individuals (Rice et al. 2015). An ideal marking material should be durable, easily applied, nontoxic (to the insect and the environment), cost-effective, and easily detectable. Also, the method of choice for applying the marker will depend on the involved insect, the environment that it will encounter, and the aim of the experiment, but essentially, it is expected that the marker should not affect the insect life span, reproduction, growth, or behavior (Hagler and Jackson 2001).

Powders (preparation in the form of fine particles) are one of the most common external markers (Silver 2008, Guerra et al. 2014) successfully used to mark adults of the genera *Aedes*, *Anopheles*, and *Culex*, mostly in mark–release–recapture (MRR) studies (Silver 2008, Johnson et al. 2017). They are offered in a wide range of colors, are cost-effective, provide an easily detected visual signal, and can be applied inexpensively to large groups of insects (Sarkar et al. 2017) in a single or multiple approaches (different contrasting colored powders in the same individual; Silver 2008). In the case of fluorescent pigments, these can be detected under longwave ultraviolet light (UV-A), without the need to destroy the specimen (Rice et al. 2015), and have been applied to mosquitoes using a variety of methods (Hagler and Jackson 2001, Neira et al. 2014). Several brands of fluorescent powders are widely used, such as Helecon, DayGlo, Hercules Radiant, LumogenTM, Fiesta, and Radglo (Silver 2008), and although no effect on mosquito survival has been found in several studies for a subset of these brands (Reisen et al. 1979, Sempala 1981, Chiang et al. 1991, Takken et al. 1998, Watson et al. 2000, Johnson et al. 2012, Liu et al. 2012, Verhulst et al. 2013), others have found increased mortality depending on the marking technique, the type of powder, or the age of the tested mosquito (Verhulst et al. 2013, Dickens and Brant 2014). Altered behavioral responses have been registered in other insect groups, such as lower response to light in *Diaphorina citri* (Asian citrus psyllids; Nakata 2008), or lower response to females in marked males of *Laspeyresia pomonella* (codling moth; Moffitt and Albano 1972).

In this study, the impact of fluorescent powders on survival, recapture by mosquito baited traps, and their detection, after being exposed to controlled laboratory and semifield settings, on females of *Ae. aegypti* was investigated. Only females were used for the experiment because of their role as vectors of pathogens and because of our goal to evaluate, for future practical uses, different brands of fluorescent powders externally applied and their impact in the aforementioned parameters in females.

Materials and Methods

Mosquitoes

Adult females of *Ae. aegypti* (Monroe County Key West Florida strain, F_{21} progeny), reared inside a climate chamber using standard protocols (Kauffman et al. 2017), were used for all experiments. Eggs were synchronously hatched in deoxygenated water prepared by use of a vacuum container powered by an electronic pump for 1.5 h. First-instar larvae were aliquoted into plastic trays (W30 × D25.5 × H4 cm) and fed daily on a combination of equal parts of brewer's yeast and lactalbumin. Female and male pupae were mechanically sorted and transferred into cups with water (Neira et al. 2014) and then into polyester mesh cages (W32.5 × D32.5 × H32.5 cm; Bugdorm insect rearing cages, BioQuip Products (California). Adults were maintained and fed with a sucrose solution (10%) renewed daily from cotton wicks. Immature stages and adults were retained inside a climate chamber (temperature: 28 ± 2°C; humidity: 80 ± 10%, photoperiod of 12:12 [L:H] h). For maintenance of the mosquito colony and collection of eggs for the experiment, adult females were fed with blood from restrained chickens (University of Florida, Animal Care and Use IACUC protocol 201003892).

Marking Mosquitoes with Fluorescent Powders of Different Brands

Seven different fluorescent powders applied externally on *Ae. aegypti* female mosquitoes were tested to analyze their influence on survival and recapture by mosquito baited traps, and their detectability after being exposed to controlled (climate chamber) and semifield conditions. The organic-based powders (in the form of fine particles) that were used are from the brands Hercules Radiant (HR), DayGlo (DG), Risk Reactor (RR), and Angstrom Technologies (AT; Table 1). Based on information provided by the manufacturers, Hercules Radiant (Brunswick, GA) powders have a triazine aldehyde amide base; DayGlo (Cleveland, OH) are incorporated into a melamine formaldehyde resin in A and Ax series (Silver 2008) or formaldehyde-free resin in ECO series; Risk Reactor (Santa Ana, CA) are combined with a melamine formaldehyde sulphonamide resin; and for Angstrom Technologies (Florence, KY), the information was not available.

Application Method of the Fluorescent Powder

Females were exposed in groups to a fluorescent powder-rich environment. Before their application, female mosquitoes were transferred to paperboard cages with mesh tops (H10 × top D10 × bottom D7 cm). Each powder was applied by filling a syringe (3 ml with needle 26G [0.45 mm]; Lapointe 2008) with the powder up to 0.5 ml (Valerio et al. 2012, Verhulst et al. 2013). A powder-rich environment was generated by gently ejecting the powder from the syringe held through the fabric mesh at the top of the cup (placed inside a plastic bag to prevent contamination of materials and working space). Following treatment, females were released from the paperboard cages into W32.5 × D32.5 × H32.5 cm polyester mesh cages.

Fluorescent Powder Detection System

Powders were detected under a stereoscope, by visual examination and under longwave UV-A light (hand-held OxyLED 51 LED 395 nm ultraviolet flashlight). The efficiency of the externally applied powders in the insect body was analyzed for each powder and scored based on a maximum 5 points rubric, where the presence of the marker was assessed independently on the head, thorax, abdomen, wings, and legs. For example, mosquitoes with detectable marking on all five parts received a 5, whereas mosquitoes with the detectable marker on only two parts received a 2 (Dickens and Brant 2014).

Table 1. Information and physical characteristics of powders used in the experiments

Experiment code	Fluorescent color	Powder ID	Brand	Mean particle size (μm)[a]	Specific gravity[a]
DG1	Fire orange	ECO 14	DayGlo	4.5	1.2
DG2	Orange	A-14-N	DayGlo	4.5–5.0	1.36
DG3	Orange	AX-14-N	DayGlo	4.5–5.0	1.36
RR1	Blue tang	PF-14	Risk Reactor	3.5–4.5	1.37
RR2	Invisible green	PF-01	Risk Reactor	3.5–4.5	1.37
HR	Red	R-103 G115	Hercules Radiant	5.0	1.4
AT	Yellow	SC-27	Angstrom Tech.	<150	N.A.

[a]Based on data provided by the manufacturer. NA, information is not available.

Experiment 1. Effect of Fluorescent Powders on Mosquito Survival

To study the effect of the seven powders on the survival of females, marked and unmarked (control) females were followed daily and the number of dead mosquitoes was recorded until all the females had died (Verhulst et al. 2013). Approximately 50 females (72 h old), maintained inside the same climate chamber, were used per treatment and controls. Two controls were used, one with females that were not handled or exposed to any powder (Valerio et al. 2012; control for the colony, sugar solution and climate-chamber conditions [Control C.]), and another one with females exposed to the marking technique but without using any powder (control of the effect of handling but not of the powder [Control A.M.]). Differences in survival between control mosquitoes and marked females with different powders were tested using a regression analysis of survival data based on the Cox proportional hazards model (PROC PHREG, SAS 9.22; SAS Institute 2010) as in other survival studies on *Ae. aegypti* (Bellamy and Alto 2018).

Experiment 2. Effect of Fluorescent Powders on Female Recapture by Mosquito Traps

The effect of the powders on female recapture by mosquito traps was evaluated after releasing them in a semifield environment and analyzing the proportion of marked females, compared with the unmarked ones, that entered a trap containing visual cues and a host odor lure (BG lure: releases lactic acid, ammonium hydrogen carbonate, and hexanoic acid; Biogents 2018). The semifield environment consisted of a double-door controlled screened enclosure (closed cylinder area [radius 712 cm, height 244 cm] with a conical roof [radius 712 cm, height 172 cm at peak]) located on the campus of the Florida Medical Entomology Laboratory in Vero Beach, FL. Experiments were performed between 20 and 30 June 2018, a period in which the average maximum and minimum reported temperature and humidity were 32.63 ± 0.74°C–23.75 ± 0.46°C and 95.00 ± 1.77%–85.88 ± 3.44%, respectively.

Each fluorescent powder was tested three times, on different days, with different combinations of brands and with 50 marked females per trial. Daily releases consisted of 100–150 exposed 5- to 11-d-old females (maximum three different markers were tested simultaneously), plus 50 unmarked females. Before the daily release of the marked and unmarked females inside the semifield environment, a BG-Sentinel trap (Biogents AG, Regensburg, Germany) was assembled and equipped with a BG lure (Biogents 2018), and a suction trap (CDC miniature trap without CO_2 nor light or any lure) was set in the opposite position of the BG-Sentinel trap as a reference trap (similar suction system, but without human odor lure and less visual cues, in comparison to the BG trap). Females were released daily at 11:30 a.m., and the traps were operated for 23:30 h. At the end of this period, each trap was collected, and caught mosquitoes were frozen and analyzed for detectable powder. Handling post-collection did not transfer powder from one individual to another. Each day, after the collection of the trap bags, mosquitoes that were flying and had not entered to any of the traps were vacuumed manually for 15 min to remove them from the environment and to ensure that their presence did not affect the subsequent release of new females marked with another powder.

A generalized linear-mixed model (GLMM) was fitted considering the number of females marked with each powder that were captured with the BG-Sentinel trap, in comparison with unmarked females that were also released and trapped. Daily powder trial was set as a random factor because unmarked females were nested across different combinations of powders every day. The relative probability of marked and unmarked groups of entering the trap was expressed as an odds ratio. The analysis was performed in R (R Core Team 2014), assuming a binomial probability distribution for the response ($\alpha = 0.05$) and a logit link function (Dorai-Raj 2014, Bates et al. 2015, Wickham 2016, Lüdecke and Schwemme 2018, Wickham et al. 2018).

Experiment 3. Detection of Fluorescent Powders on Marked Females

The detection of powders in marked females was analyzed for 30 d under controlled (climate chamber) and semifield conditions. For testing each powder, 100 ± 10 females (48–72 h old) were treated and placed inside polyester mesh cages in each environment. Every 24 h, three females per treatment were randomly collected, frozen, and examined with the detection and score system described earlier.

Cages under the controlled environment (climate chamber) were maintained with a temperature of 28 ± 2°C, humidity of 80% ± 10%, and a photoperiod of 12:12 (L:D) h. Cages set under semifield conditions were placed in a natural environment exposed to weather conditions and on top of a wooden pallet with each leg placed on a tray with soapy water to prevent access by ants. Cages were placed under eaves and so were partially protected from rainfall. A black plastic cup was set inside each cage, under both conditions, to provide a mosquito resting site (Facchinelli et al. 2011, Valerio et al. 2012). Weather conditions (temperature, humidity, wind speed, and direction) were recorded daily with an AcuRite high-precision 5-in-1 weather sensor placed near the cages. A descriptive analysis of the data, by powder and surrounding conditions, was performed.

Results

Experiment 1. Effect of Fluorescent Powders on Mosquito Survival

Survival distributions, among marked and unmarked females, were not significantly different from one another ($\chi^2 = 10.54$, df = 8, $P = 0.229$; Fig. 1). In total, 531 females were used to measure treatment effects on survival.

Experiment 2. Effect of Fluorescent Powders on Female Recapture by Mosquito Traps

Even though mean percentages of caught marked females with all powders were above 50%, these females were significantly at lesser odds of entering the baited trap in comparison to unmarked ones for most of the powders. The highest proportion was achieved by the females marked with DG1 and the least one by those marked with AT (Fig. 2). Only six females, scattered among powders or unmarked females and between days, were collected by the reference trap. Marking on just one part of the body with a different powder that the one present or absent in the rest of the body, indicating a potential powder transfer between individuals, was not observed in any of the specimens.

The GLMM showed that females marked with powder DG1 were the only ones that were not significantly at lesser probabilities of entering the trap in comparison to unmarked ones (Table 2).

Experiment 3. Detection of Fluorescent Powders on Marked Females

Most of the markers could be detected on all females until day 30, under both environments. Only for powder RR2 did the percentage

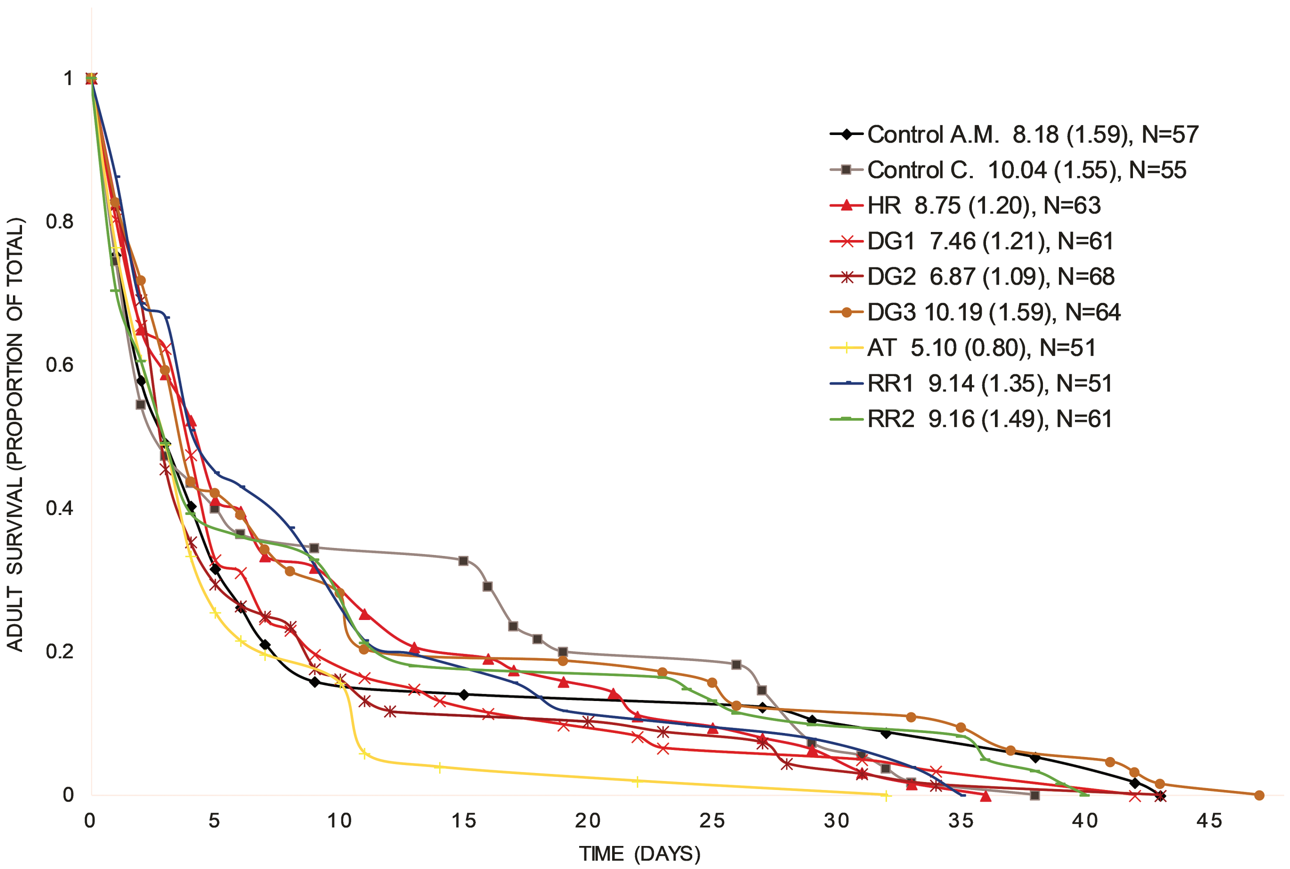

Fig. 1. Effect of fluorescent powders of different brands on the survival of *Aedes aegypti* females. Numbers following the powder names indicate mean (± SE) lifespan in days, and the total number of females sampled (N) per powder. Females were marked with fluorescent pigments of different brands (Hercules Radiant [HR], DayGlo [DG], Risk Reactor [RR], and Angstrom Technologies [AT]). Control of the application method (Control A.M.) and Control Colony (Control C.).

of females with a score of 5 vary after day 11 and, was 61 and 48% under controlled and semifield conditions, respectively. Marked females that lost the powder tended to lose it more promptly from wings and legs, and all of them remained marked in head and thorax (the lowest score was 2). The loss was not gradual in all the females because some of them presented a score of 5 on day 30.

Average temperature and humidity were similar under controlled (temperature 27 ± 0.3°C, humidity 75 ± 4.4%) and uncontrolled conditions (temperature 28 ± 4.8°C, humidity 88 ± 10.2%), but with higher values and data dispersion in the semifield environment.

Discussion

Evaluating the practical features of research tools, such as insect markers, is fundamental for using such tools to make inferences from experiments. Although fluorescent pigments in their powdered form have been frequently used as mosquito markers (Silver 2008), few studies have directly assessed their effects on the survival or behavior of females of *Ae. aegypti* or their detectability after being exposed to different environmental conditions.

Several brands of fluorescent powders have been used in studies of *Ae. aegypti*, frequently in MRR studies, with DG and bulb-dusters or syringes as brand and method of application most commonly used (Muir and Kay 1998, Harrington et al. 2005, Maciel de Freitas et al. 2007, Maciel de Freitas and Lourenço de Oliveira 2009, Valerio et al. 2012, Dickens and Brant 2014, Mondal et al. 2017). Like most of these studies, no significant differences in survival distributions among marked and unmarked females among any of the brands while using the described marking technique were observed in this research. These findings support their value as markers and point out new available brands that may also function under this experimental scheme. One detail that should be considered is the lack of evidence for the influence of fluorescent powders on the survival of adults when using 2- or 3-d-old females of different species, such as *Ae. aegypti* (Johnson et al. 2012, Dickens and Brant 2014), *Aedes africanus* (Sempala 1981), *Aedes notoscriptus* (Watson et al. 2000), and several species of *Anopheles* (Reisen et al. 1979, Chiang et al. 1991, Takken et al. 1998, Liu et al. 2012, Verhulst et al. 2013). In contrast, negative effects were reported in *Anopheles*, when treated at an older age (Verhulst et al. 2013). More detailed effects on this important parameter of the vectorial capacity index should be investigated across ages in the vector of interest, considering that age-dependent mortality may increase with age (*Ae. aegypti*; Styer et al. 2007a,b; Harrington et al. 2008) or interact with other factors, such as temperature, reproductive status, and cumulative blood feeding (*Anopheles stephensi* [Dawes et al. 2009], *Aedes albopictus* [Leisnham et al. 2008]).

In regard to the effect of marking on the female recapture by mosquito traps, even though more than the half of the released marked females entered the baited trap, and almost none of them entered the reference trap, they were less prone to enter the baited trap compared with unmarked females for most of the powders. This may be due to interference with sensory organs related to olfaction, such as sensilla located in various head appendages (e.g.,

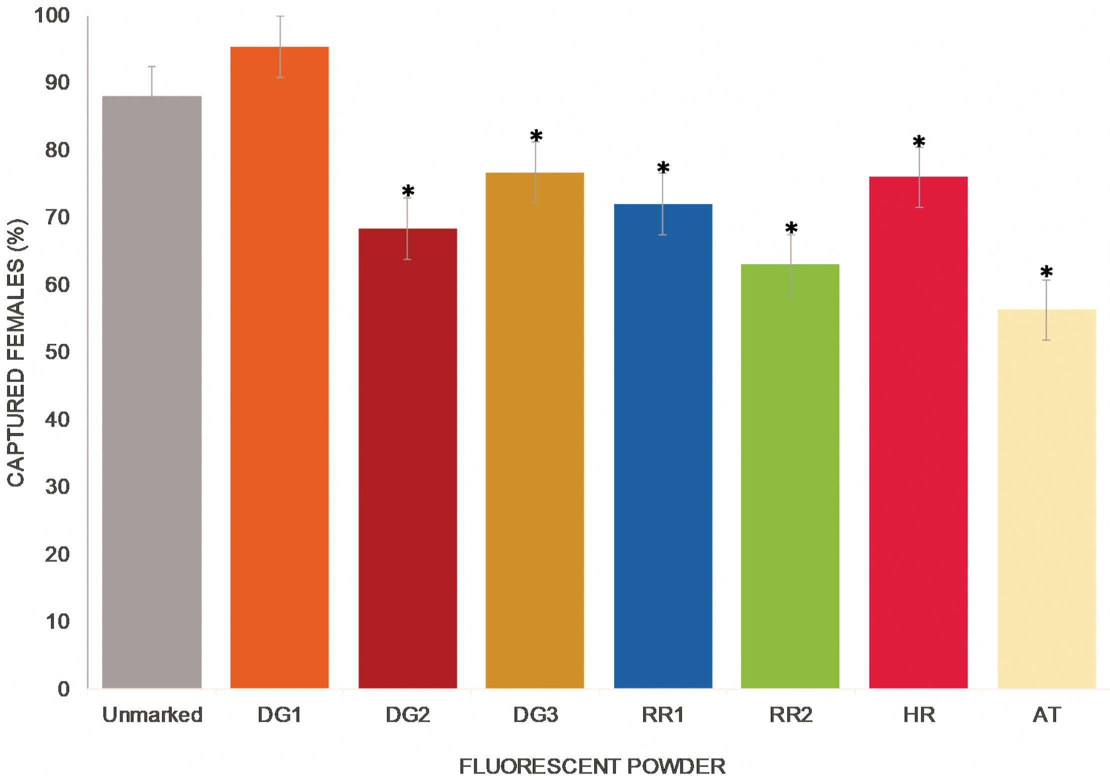

Fig. 2. Effect of fluorescent powders on *Aedes aegypti* female recapture by mosquito traps. Mean proportions (based on the total released number) of marked and control females that entered a baited BG-Sentinel trap. Error bars represent SE, and asterisk denotes significant differences between treatments and unmarked females as determined by the GLMM. Females were marked with fluorescent pigments of different brands (Hercules Radiant [HR], DayGlo [DG], Risk Reactor [RR], and Angstrom Technologies [AT]).

Table 2. Statistical analysis of the ratio between the number of unmarked and marked females that were released and entered the baited trap

Powder	Odds ratio (95% CI)	*P*
Unmarked[a]	1	
DG1	2.34 (0.99–5.52)	0.052
DG2	0.36 (0.22–0.60)	<0.001
DG3	0.41 (0.23–0.70)	0.001
RR1	0.31 (0.18–0.54)	<0.001
RR2	0.29 (0.17–0.47)	<0.001
HR	0.34 (0.20–0.60)	<0.001
AT	0.30 (0.19–0.47)	<0.001
σ^2		3.29
Marginal R^2		0.12
Conditional R^2		0.22

[a]Reference level.

antenna, maxillary palp, and labellum; Takken and Knols 2010), mosquito eyes, mechanoreceptors, or an effect on other physiological processes that impedes flight toward the trap in the same time frame as the unmarked females. This contrasts with the findings in *Anopheles gambiae*, where no effect on sensory organs was found in marked females using a dual-port olfactometer with human or cow odor (Verhulst et al. 2013). More experiments should be done with several formulations of powders because in this experiment, females marked with DG ECO series, which in contrast to the rest of powders is incorporated into a formaldehyde-free resin and has a lower specific gravity, presented no difference in the odds of entering the trap compared with unmarked females. It is important to highlight the usage of lure-baited BG-Sentinel traps to indirectly evaluate the host-seeking performance of *Ae. aegypti* females because this trap uses a combination of female attractive compounds found on human skin (Biogents 2018), aided with visual cues that give additional information about the potential impact of these powders in other sensory organs (Ball and Ritchie 2010, Biogents 2018). Some limitations of this approach are the fact that the trap has both visual and olfactory attractants, so it is not possible to discriminate whether none, only one, or both senses can be affected by markers and that despite the deliberate use of a host odor lure, it is possible that BG traps may collect other physiological stages of *Ae. aegypti* females. Another limitation in this study was that the traps were not swapped into different positions for evaluating the position effect.

The detectability of the powder on treated females was consistent among most of the brands under controlled and semifield conditions during the observed period, suggesting that these powders and the marking method are appropriate for tracking mosquitoes for a considerable period of their adult life. These results are similar to the observations reported by other researchers (Nelson et al. 1978, Muir and Kay 1998, Verhulst et al. 2013, Dickens and Brant 2014). The powder that did not remain with a score of five throughout the observation period, unlike the others, was the only one that could not be seen with the naked eye and can only be observed with ultraviolet light (hence its name is invisible green). It is possible that this specific formulation has lower adherence to the mosquito cuticle. Another important aspect is that no difference was observed between both conditions (controlled and semifield), which indicates that the presence of sunlight, a wider range of humidity and temperature, and other environmental factors do not affect the permanence and detection of these powders. The effect of direct rain was not tested in this study, mainly because *Ae. aegypti* is an anthropophilic, endophilic, and endophagic mosquito (Christophers 1960, Reinhold et al. 2018) that usually remains in the peridomestic environment and uses refugee or resting sites (Maciel de Freitas et al. 2006) under certain weather conditions. The effect of precipitation can be tested in future studies because it is expected that the detectability may be lower under that condition in the field.

The fact that mosquitoes do not easily lose powder from their head and thorax, against their wings and legs, has also been reported previously (Bennet et al. 1981). This may be correlated with the fact the mosquitoes tend to groom primarily their legs and wings (Supp Video S1 [online only]) in sequences that involve tibia comb-like structures (Goldman et al. 1972) and comprises different leg parts and wings dorsal and ventral surfaces (Walker and Archer 1988). This behavior may have an evolutionary advantage because clean legs and wings could play a role in sensory functions and flight, respectively (Jacquet et al. 2012).

Careful examination of the markers used in experiments should be done with the species of interest and from different approaches because their practical use and any conclusions derived from them in a study are only possible if their limitations and characteristics under specific conditions have been investigated and recognized. The lack of evidence that the presence of the powders affects mosquito survival and the detectability of most of the tested brands throughout the mosquito lifespan makes them a useful tool for studies that need to mark *Ae. aegypti* for different purposes, although more studies of their impact on the host-seeking activity using live hosts and different trap displays (e.g., with and without lure or traps of different colors) to distinguish which sensory organs may be potentially affected should be performed. Future studies may focus on the effect of these markers on survival across different ages of mosquitoes, blood-feeding and mating behavior, dispersal traits, natural fluorescence of the insect involved, and potential bias related to this marking technique, such as the possible contact transfer from marked to unmarked individuals.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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