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SCIENTIFIC NOTE

EFFECTS OF A RED MARKER DYE ON *AEDES* AND *CULEX* LARVAE: ARE THERE IMPLICATIONS FOR OPERATIONAL MOSQUITO CONTROL?

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ABSTRACT. Marker dyes are often mixed with liquid insecticide formulations prior to field applications to accurately determine the characteristics and penetration of droplets into targeted habitats. We have been using FD&C Red 40 Granular DM food dye at the rate of 20 g/liter in liquid solutions of *Bacillus thuringiensis israelensis* (*Bti*) for area-wide larvicide applications against the Asian tiger mosquito, *Aedes albopictus*. The *Bti* and dye mix ratio has been recommended by pesticide manufacturers for testing under operational conditions, but no data exist on the effects of the dye itself on mosquito larvae. We tested the effects of the FD&C Red 40 food dye in laboratory bioassays against different strains of *Ae. albopictus* (New Jersey and Maryland) and *Culex pipiens pipiens* (Utah) at rates of 0.039 to 80.0 g/liter. We also conducted field application trials to measure dye concentrations up to 100 m downwind when mixed and applied according to manufacturer instructions. In laboratory bioassays, we found that mean survival in cups with dye were significantly different from the controls beginning at 10.0 g/liter for New Jersey *Ae. albopictus* and at 20.0 g/liter for Maryland *Ae. albopictus* and Utah *Cx. p. pipiens*. In field application trials, we recorded a maximum volume density of 1,152.8 nl/cm^2 and calculated the maximum concentration of dye at 9.09×10^{-3} g/liter. Our results showed that although we detected greater effects of dye on *Ae. albopictus* in New Jersey experiments than *Ae. albopictus* in Maryland and *Cx. p. pipiens* from Utah, concentrations of the dye during operational applications were at least 1,100 times below concentrations that exhibited toxic effects for either species in the laboratory, suggesting that the dye will not interfere with accuracy of field bioassays. Our results conclusively demonstrate that the addition of the FD&C Red 40 marker dye does not alter the efficacy of the pesticide formulation by skewing results, but rather provides a valuable addition to accurately determine pesticide penetration and spectrum by discriminating between intended pesticide and other potential pollutants.

KEY WORDS *Aedes albopictus*, *Culex pipiens*, larval mortality, low-volume larvicide, marker dye

Testing insecticide application equipment and methodology is integral in developing the most efficient and effective management tools to control mosquito populations. Because invasive *Aedes albopictus* (Skuse) are ubiquitous in many urban environments worldwide, area-wide insecticide applications are often the most effective means of population control and minimizing vector-borne public health threats, including dengue and chikungunya viruses (WHO 2009, Farajollahi et al. 2012, Rochlin et al. 2013). For such applications to be successful in urban landscapes that are often difficult to access and full of cryptic container habitats, it is imperative to determine the characteristics such as droplet density, size, and penetration of the product into the various habitats (Arredondo-Jimenez and Rivero 2006, Sun et al. 2014). A marker dye is generally used in operational trials with formulations of liquid insecticides to determine droplet characteristics (size and density) and penetration (Britch et al. 2010).

Pesticide manufacturers often recommend a FD&C Red 40 Granular DM food dye (Glanbia Nutritionals, Carlsbad, CA) to be used with liquid larvicides to determine droplet characteristics. Mercer County Mosquito Control has previously utilized this dye with the bacterial agent *Bacillus thuringiensis israelensis* de Barjac (*Bti*) for multiple field applications against *Ae. albopictus* (Williams et al. 2014). The water-dispersible granular formulation of *Bti*, VectoBac WDG® (Valent Biosciences, Libertyville, IL), mixes readily with water for low-volume larvicide applications to target urban populations of peridomestic *Aedes* mosquitoes. In previous laboratory trials, VectoBac WDG has exhibited excellent mortality against container-inhabiting *Aedes* (Farajollahi et al.

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2013); however, no data exist on the effects of the food dye itself on mosquito larvae. In this study, we tested the effects of the FD&C Red 40 food dye in the field and in laboratory bioassays against different strains of *Ae. albopictus* and *Culex pipiens pipiens* L.

To determine if FD&C Red 40 Granular DM food dye causes larval mortality, *Ae. albopictus* larvae from New Jersey and Maryland, and *Cx. p. pipiens* larvae from Utah were used. *Aedes albopictus* larvae were used from established laboratory colonies that were regularly supplemented by local field collections (F_{1-3} generation). *Culex p. pipiens* larvae were hatched from field-collected egg rafts.

Bioassays for each strain were conducted separately at different times, but followed the same protocol. Larvae were reared in trays containing 2 liters of dechlorinated tap water at 27°C under a 16:8 h light:dark photoperiod. Developing larvae were fed finely ground rat chow (0.5 g dissolved in 50 ml of water added to rearing tray) and all rearing trays were skimmed daily to remove waste buildup on the surface of the water. Only 3rd-stage larvae were used for experiments.

Manufacturers recommend FD&C Red 40 Granular DM to be mixed at a rate of 20 g/liter (2% weight/volume) for all operational trials (P. DeChant, Valent BioSciences, personal communication). We used 12 dye concentrations (0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 g/liter) and a control concentration with no dye for all laboratory experiments. The tested concentrations allowed for an approximate range likely to penetrate larval container habitats via direct field applications. The dye was weighed with a digital scale and added to 16-oz (473 ml) disposable polystyrene cups (Solo Cup Company, Lake Forest, IL) containing 400 ml of dechlorinated tap water. The contents in each cup were manually stirred with a wooden applicator stick to completely dissolve the dye. Third-stage larvae were removed from rearing trays using disposable plastic pipettes and randomly added to experimental cups. A total of 10 (New Jersey *Ae. albopictus*) or 15 (Maryland *Ae. albopictus*, Utah *Cx. p. pipiens*) larvae were added to each cup. For each strain, the 13 test concentrations were replicated 4–6 times, depending on availability of larvae. Larval food was provided to experimental cups in the same concentration as in rearing trays. Because larvae were difficult to visually inspect at the higher dye concentrations, liquid contents of the bioassay cups were emptied into white trays to assess mortality and then returned. To avoid contamination, trays were rinsed between cups and separate trays were used for control cups and those with dye. Cups and larval morality were examined and recorded daily. Larvae were considered dead if there was no response to gentle prodding with a pipette tip, and were then removed and disposed of.

The survival of mosquito larvae exposed to varying concentrations of red dye was examined using PROC LOGISTIC (SAS version 9.3 for Windows SAS Institute, Cary, NC). Model 1 was used to estimate the proportion of survival as a function of dye concentration, treating concentration as a categorical predictor. Post hoc tests (Bonferroni correction) were conducted to compare each of the 12 dye concentrations with the control. The Holm's test was used to control for multiplicity (alpha = 0.05) (Hochberg 1988). The model was then refit using concentration as a continuous predictor to estimate concentrations that would result in 75%, 50%, 25%, and 5% survival (Model 2).

We fitted Model 1 using the default logit link for all 3 experiments (New Jersey, Utah, and Maryland). Firth's penalized likelihood (Fort and Lambert-Lacroix 2005) was used to compensate for quasi-complete separation in the New Jersey and Utah models. Overdispersion was estimated using the Williams' method and was detected in the Maryland (Pearson $\chi^2/df = 2.76$ and Utah (Pearson $\chi^2/df = 1.88$) models (Farrington 1992). We fitted Model 2 using the complementary log-log link for all models. Overdispersion was detected in the Maryland (Pearson $\chi^2/df = 2.55$) and Utah (Pearson $\chi^2/df = 3.23$) models.

Concentration was a significant predictor of larval survival for all 3 models (New Jersey: $\chi^2 = 177.77$, df = 12, $P < 0.0001$, max-rescaled $R^2 = 0.899$; Utah: $\chi^2 = 62.32$, df = 12, $P < 0.0001$, max-rescaled $R^2 = 0.773$; Maryland: $\chi^2 = 72.51$, df = 12, $P < 0.0001$, max-rescaled $R^2 = 0.664$) (Table 1). Mean survival in cups with dye were significantly different from the control beginning at 10.0 g/liter for New Jersey and 20.0 g/liter for Maryland and Utah.

While the dye was found to have some toxic effects on *Ae. albopictus*, operational concentrations of dye are too low to affect larval mortality. Previous experiments determined the droplet densities during field applications of *Bti* with a CSM2 Buffalo Turbine mist sprayer and a Curtis Dyna-Fog LV8 orchard sprayer (Williams et al. 2014). In short, 20 g/liter of FD&C Red food dye mixed with VectoBac WDG was sprayed at rates of 400 and 800 g/ha. Bioassay cups and Kromekote cards (CTI Paper, Sun Prairie, WI) were placed out to 100 m from the point of application. Volume density was recorded from the cards utilizing the DropVision® AG system (Leading Edge Associates, Inc., Waynesville, NC). The maximum volume density recorded during the field trials was 1,152.8 nl/cm^2, but most densities were much lower (mean 27.7; range 0–1,152.8; SE 5.2 nl/cm^2) (Williams et al. 2014). The maximum volume density was extrapolated to determine the maximum concentration of dye delivered during

Table 1. Observed survival and estimated probability of survival with 95% confidence limits (upper and lower control limits)[1] for 3rd instars of mosquitoes exposed to varying concentrations of red dye.

Species		Concentration of red dye (g/liter)												
		No dye	0.039	0.078	0.156	0.313	0.625	1.25	2.5	5	10	20	40	80
Aedes albopictus (New Jersey)	No. alive per trial (n = 10)	10, 10, 10, 10, 10, 10	10, 10, 10, 10, 10, 10	10, 10, 9, 10, 10, 10	10, 10, 10, 10, 10, 10	10, 10, 8, 10, 10, 10	10, 10, 9, 10, 10, 9	10, 8, 9, 10, 10, 9	9, 10, 10, 9, 8, 10	10, 9, 7, 8, 9, 7	7, 6, 7, 5, 3, 2	4, 5, 0, 1, 1, 0	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
	Est. prob.	0.99	0.99	0.98	0.99	0.96	0.96	0.93	0.93	0.83	0.50*[2]	0.19	0.01*	0.01*
	LCL	0.88	0.88	0.89	0.88	0.87	0.87	0.83	0.83	0.71	0.38	0.11	0.00	0.00
	UCL	1.00	1.00	1.00	1.00	0.99	0.99	0.97	0.97	0.90	0.62	0.31	0.12	0.12
Culex pipiens pipiens (Utah)	No. alive per trial (n = 15)	14, 13, 10, 13, 10	15, 14, 15	15, 12, 12	3, 13, 1	12, 14, 8	13, 13, 13	9, 12, 12	12, 10, 12	12, 9, 11	10, 6, 9	0, 0, 2	0, 0, 0	0, 0, 0
	Est. prob.	0.79	0.96	0.85	0.38*	0.75	0.85	0.72	0.75	0.70	0.55	0.06*	0.02*	0.02*
	LCL	0.64	0.76	0.65	0.21	0.54	0.65	0.52	0.54	0.50	0.36	0.01	0.00	0.00
	UCL	0.89	0.99	0.95	0.59	0.88	0.95	0.87	0.88	0.85	0.73	0.26	0.26	0.26
Ae. albopictus (Maryland)	No. alive per trial (n = 15)	15, 14, 15, 11	13, 10, 14, 15	15, 14, 15, 10	13, 13, 9, 15	14, 14, 15, 10	13, 10, 14, 14	11, 15, 15, 14	13, 15, 13, 14	13, 8, 14, 14	12, 13, 10, 3	5, 2, 5, 4	2, 4, 0, 2	0, 0, 0, 2
	Est. prob.	0.92	0.87	0.90	0.83	0.88	0.85	0.92	0.92	0.82	0.63	0.27*	0.13*	0.03*
	LCL	0.71	0.65	0.69	0.62	0.67	0.64	0.71	0.71	0.60	0.42	0.12	0.04	0.00
	UCL	0.98	0.96	0.97	0.94	0.97	0.95	0.98	0.98	0.93	0.81	0.48	0.35	0.26

[1] Est. prob., Estimated probability; LCL, lower confidence limit; UCL, upper confidence limit.
[2] * denotes significant differences.

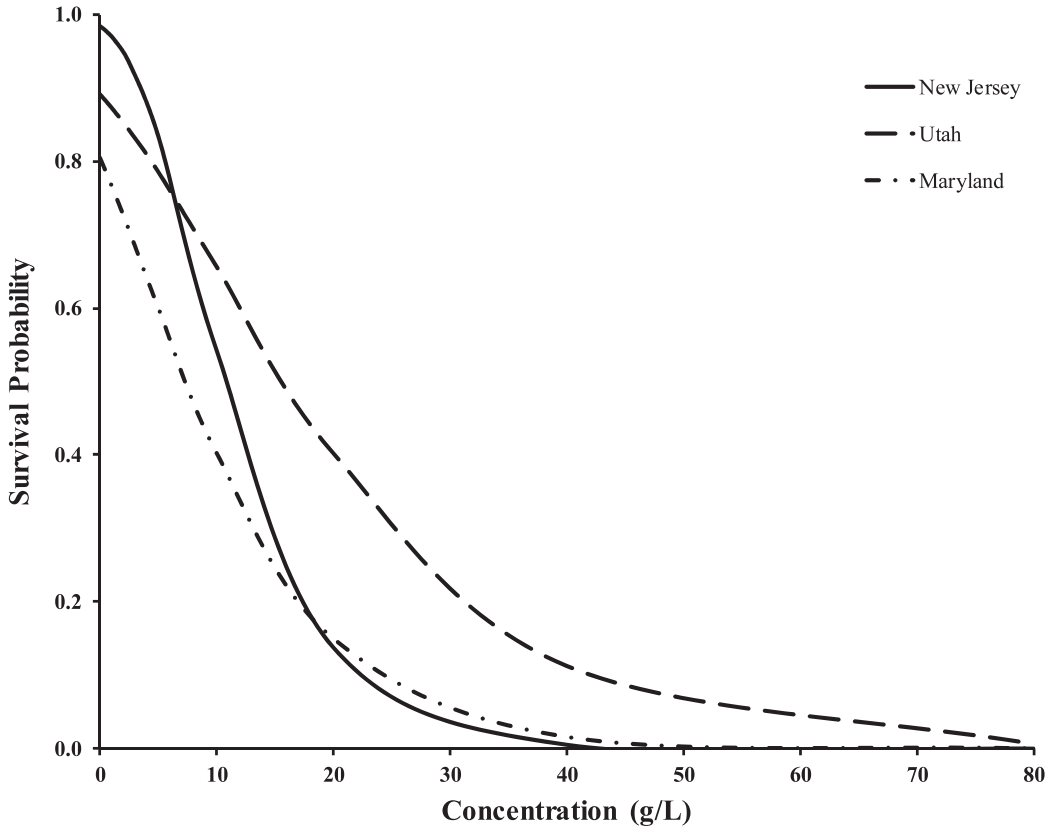

Fig. 1. Probability of survival for 3rd instars of *Aedes albopictus* and *Culex pipiens pipiens*.

the trials based on the surface area of the bioassay cups. With a 2% mix of dye and 250 ml volume of water, the maximum concentration of dye recorded was 9.09×10^{-3} g/liter. This amount is 1,100 times lower than the minimum toxic levels reported here (Fig. 1).

In conclusion, the food dye FD&C Red 40 Granular DM showed significant mortality over the control containers in laboratory test conditions. In field trials the amount of dye delivered to the containers was minuscule and diluted, indicating that it will not bias larval mortality. We conclude that adding FD&C Red 40 Granular DM to formulations of VectoBac WDG at the concentrations that we tested is a suitable marker to use during operational applications to determine the characteristics and penetration of droplets.

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