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SCIENTIFIC NOTE

ULTRA-LOW VOLUME APPLICATION OF SPINOSAD (NATULAR 2EC) LARVICIDE AS A RESIDUAL IN A TROPICAL ENVIRONMENT AGAINST *AEDES* AND *ANOPHELES* SPECIES

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ABSTRACT. We investigated the efficacy of a liquid larvicide, Natular 2EC® (spinosad), applied with ultra-low volume sprayer as a residual application during the dry season in southeastern Thailand against 4 medically important species—*Aedes aegypti*, *Ae. albopictus*, *Anopheles dirus*, and *An. minimus*. We found that this larvicide could be applied as a residual to dry areas known to collect water and potentially still be effective after rains or irrigation, which could increase the flexibility and efficiency of an integrated vector management program targeting these species. This investigation also demonstrated, for the 1st time, efficacy of spinosad against *An. minimus* and *An. dirus*.

KEY WORDS Biorational, container inhabiting, forest, peridomestic, pretreatment

In tropical regions such as Thailand, endophilic and peridomestic mosquitoes, such as *Aedes aegypti* (L.), and sylvatic mosquitoes that can occur near human settlements, such as *Ae. albopictus* (Skuse), *Anopheles dirus* Peyton and Harrison, and *An. minimus* Theobald, exploit abundant, cryptic, and often minute pockets of standing water for larval development (Kittayapong and Strickman 1993, Rattanarithikul et al. 1995). These larval habitats as a whole are effectively impossible to identify, eliminate, and treat by hand with larvicides. They can be treated at the area level in part using liquid larvicides applied, for instance, with ultra-low volume (ULV) spray equipment. Unfortunately, mosquito control resources are typically limited and it may not always be possible for integrated vector management (IVM) actions such as ULV larvicide applications to be synchronized with high-risk periods of mosquito development. One improvement to the IVM process could be to develop larviciding techniques to pretreat areas while dry that are rich in habitat that tends to collect standing water.

We conducted a field study to investigate efficacy of Natular 2EC®, a liquid larvicide, applied with ULV as a residual during the dry season (April 2016) in Soi Dao District, Chanthaburi Province, in southeastern Thailand in areas of natural vegetation within a rubber tree (*Hevea brasiliensis* Muell. Arg.) plantation (13.051789°N, 102.284323°E; approximately 165 m in elevation). The active ingredient of Natular 2EC is spinosad, a biological larvicide formulated from a mixture of spinosyn A and spinosyn D, which are products of fermentation by the bacterium *Saccharopolyspora spinosa* Mertz and Yao that cause muscle convulsions, paralysis, and eventually death in susceptible mosquito larvae that consume or directly contact the formulation (Hertlein et al. 2010, Kirst 2010). Spinosad has documented efficacy against *Ae. aegypti* (Darriet et al. 2005) and *Ae. albopictus* (Marina et al. 2011), and efficacy has been documented in select *Anopheles* species (e.g., *An. albimanus* Weid.; Bond et al. 2004) but not yet in *An. dirus* or *An. minimus*. In an earlier study in Florida we determined that efficacy of Natular 2EC could be equally assessed in the field by capturing droplets in empty 473-ml plastic cups and later adding water and sentinel colony-reared *Ae. aegypti* larvae, compared with capturing droplets in cups already containing water and larvae (see Aldridge et al. 2018, this issue). Aside from the obvious advantage of not having to maintain sentinel larvae in the field, this finding led us to consider the possibility that Natular 2EC could be applied to dry natural habitats as a residual and effectively impact mosquito populations when these areas were later flooded.

In the present study, 3 trials were conducted with Natular 2EC in water at the maximum label rate of 2.8 fl oz/acre (204.6 ml/ha) applied with a Twister XL3 (45 ml/min flow rate; median droplet size (DV_{50}) <15 μm across a range of flow rates up to 157 ml/min; Curtis Dyna-Fog, Westfield, IN) ULV backpack sprayer targeted at a series of empty 473-ml plastic sentinel larvicide collection cups (DeliPRO TD40016; TriPak Industrial USA, LLC, White Plains, NY) placed either in the open or under low vegetation canopy in the rubber tree plantation. In this plantation, abundant larval habitat is present in the form of standing water pooling in discarded sap

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pots and a variety of small glass and plastic containers strewn throughout the vegetation. The plastic 473-ml sentinel cups simulated this local larval habitat in a standardized way.

We delineated a 60-m east–west transect along the north side of a dirt road where the 1st 30-m segment ran alongside dense vegetation canopy of forbs approximately 0.25–1 m tall immediately adjacent to the road and the 2nd 30-m segment ran by sparse, open vegetation. Previous trials demonstrated that the visible ULV spray traveled approximately 6–7 m from the Twister operator, producing approximately 0.1 acre (0.04 ha) of larvicide coverage for the 60-m transect. The beginning of each 30-m segment and every 5 m thereafter was marked with pin flags ("stations") placed about 1 m north of the road. We placed clusters of 8 sentinel cups at each of the 2nd through 6th stations in the dense canopy area and similarly at the 8th through 12th stations in the open area. The cups were thus positioned so that the ULV spray was flowing at least 5 m before and after the stations to increase likelihood of contact of all sentinel cups with larvicide given normal variations in wind speed and direction expected during the application. For each trial the spray operator initiated the ULV spray and began walking west to east along the road from the transect starting point at a pace of approximately 12 sec for each 5-m distance, totaling 144 sec to walk 60 m to the ending point where the machine was turned off. The operator maintained a distance of approximately 1–2 m from the vegetation/road boundary during the entire walk. Prevailing winds were from the south to south-southeast throughout the experiment, generally pushing the ULV spray from the walking line into the sentinel cup stations to the north of the dirt road. Three trials were conducted on separate, consecutive days April 6, 7, and 8. We established an untreated control area with sentinel cups about 50 m upwind from the end of the transect.

At each station we deployed a cluster of 8 labeled sentinel cups for each spray trial to provide 2 cups for bioassays for each of 4 mosquito species—*Ae. aegypti*, *Ae. albopictus*, *An. minimus*, and *An. dirus*. At the control area we deployed 48 labeled sentinel cups (24 in an open area, 24 under dense vegetation canopy) for each spray trial to provide 12 control replicates (6 open, 6 canopy) for each species. Once the operator ended the ULV application, we left all cups in place for a 10-min hold time to allow ULV droplets to settle and then capped and collected all cups into large black plastic bags. These bags were stored in the shade while in the field but brought into an air-conditioned room later in the day, and eventually transferred in an air-conditioned vehicle to a laboratory environment at the Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok until colony larval specimens were available to initiate bioassays starting in late April 2016, approximately 3 wk after the field applications. Once colony larvae became available, half of the cups from each habitat type for each species across the 3 trials were subjected to the bioassay protocol right away. The other half of the cups were left uncovered in the laboratory environment for 7 days before initiating bioassays to investigate effects of prolonged exposure to light and air circulation on efficacy of the Natular 2EC residual.

The bioassay protocol consisted of removing the lids from the sentinel cups and introducing 400 ml of distilled water from the AFRIMS insectary water supply and 50 2nd and 3rd instars of 1 of the 4 tested mosquito species. *Anopheles dirus* and *An. minimus*, major vectors of human malaria in Thailand, were sampled as larvae and pupae from Chanthaburi in 1985 and Tak in 1990, respectively. *Aedes aegypti* and *Ae. albopictus* were originally collected from field sites in Bangkok and Krabi, respectively, in 1992. All mosquito species were reared at 27 ± 2°C at 80 ± 10% RH under a photo regime of 12:12 h (L:D) provided by 35-W fluorescent lights in the AFRIMS insectary. Larvae in each sentinel cup were fed with 30 mg fish food (C.P. Hi Pro®; Perfect Companion Group Co., Ltd., Bangkok, Thailand). These cups were then covered with emergence funnels (1425 Mosquito Breeder; Bioquip, Rancho Dominguez, CA) and placed in incubators under the same environmental parameters as colony rearing. To determine efficacy of the residual larvicide we recorded the number of adult mosquitoes emerging from each cup until no additional adults emerged, up to 10 days postintroduction. From these data we calculated the percent mortality in each cup as $100 \times [(50 - A)/50]$, where A is the number of emerged adults from each cup.

Mortality data were analyzed with Wizard 1.9.8 (Evan Miller, http://www.wizardmac.com; Chicago, IL); all mortality data were Abbott-corrected (Abbott 1925) prior to analysis. Significant variation in mortality was observed from trial to trial for some species among cup stations in each habitat type (ANOVA data not shown). However, this was expected due to the natural variations in wind currents that were observed as the wind passed through the dense stand of rubber trees throughout the 3 trials and was ignored by combining mortality data for each species across stations. For most species in both habitats, mortality in Trial 1 was significantly lower than in Trials 2 and 3 (ANOVA data not shown) most likely due to variation in winds. Trials were conducted in the morning between 1000 h and 1200 h each day. The spray operator walked west to east to best use the steady, low wind (approximately 2–3 mph, approximately 92°F, approximately 50% RH) from the south-southeast in Trial 1. However, for Trials 2 and 3, the wind was more directly from the south and faster with more gusts (approximately 3–7 mph, approximately 91–93°F, approximately 47–54% RH). We also suspected a possible flow rate problem with the ULV sprayer during Trial 1. The exception was *An. minimus*,

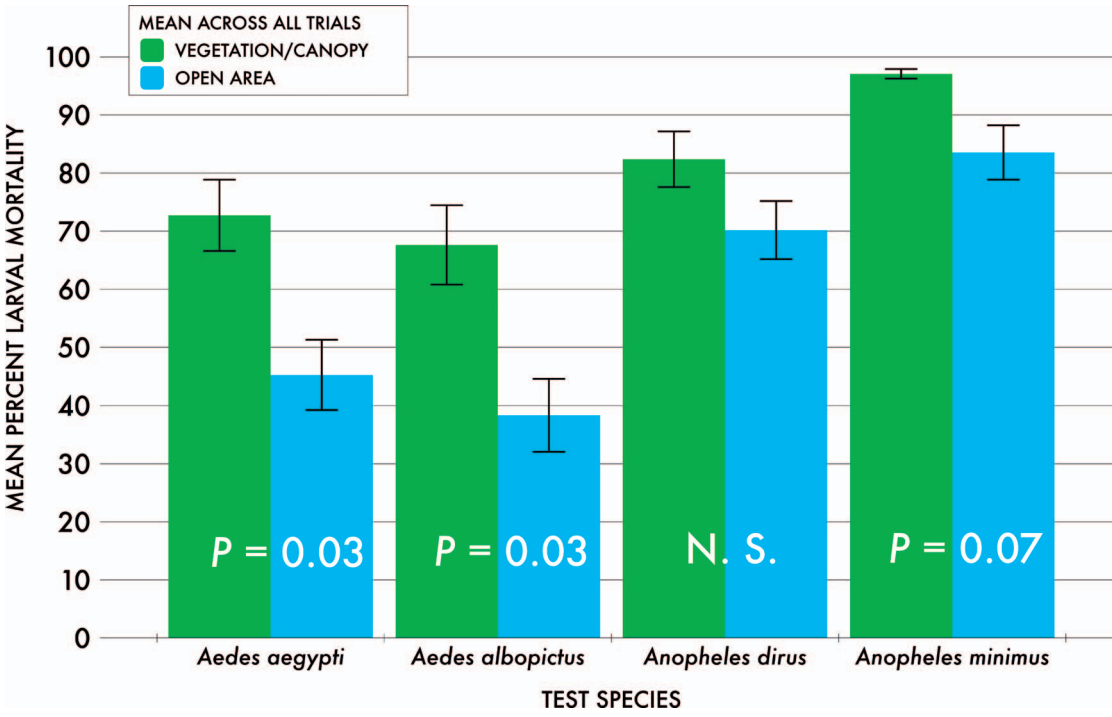

Fig. 1. Mean percent mortality from exposure to spinosad pooled across 3 trials among the 4 tested species by habitat type. A standard error of the mean is indicated on each histogram. The *P*-value for each species is derived from *t*-test analyses comparing the effect of sentinel cups placed under heavy vegetation canopy to cups placed in open areas. Mortality was significantly higher ($P \leq 0.007$) under canopy compared with open areas for all species except *Anopheles dirus* ($P = 0.089$).

which was the most sensitive of the tested species and showed uniformly high mortality across all 3 trials. Nevertheless, we ignored these differences by combining data for each species across trials. The main analyses we conducted investigated effects of habitat type (vegetation canopy versus open area; *t*-test) and bioassay interval (i.e., 0 days versus 7 days after cups uncovered; *t*-test) for each species and across all species combined.

We found no effect of interval on the outcome of mortality in bioassays ($0.600 \geq t(58) \geq 0.034$; $0.956 \geq P \geq 0.551$; data not shown): waiting another week to do bioassays with the cups uncovered in the lab and subject to evaporative processes made no difference. This is perhaps not surprising since the set of bioassays conducted immediately after the cups were uncovered were themselves about 3 wk after the field trial. The cups had been covered and stored in darkness in a cool, dry indoor location before bioassays, but we hypothesize that this could not have completely prevented evaporation of volatile components of the minute quantities of larvicide into the air pocket in the cup, which would have been drawn out into ambient atmosphere as soon as the cover was removed. Regardless, the fact that Natular 2EC applied under field conditions could still be bioactive following extended storage was encouraging for further study of its operational use as a residual. In future investigations we plan to expose uncovered cups with field-applied Natular 2EC to more real-world weathering conditions after storage, for instance placing them in an outdoor location protected from rain but subject to insolation and circulation of air, humidity, and airborne particulates.

After having found no effect of interval on mortality, we pooled percent mortality data from both intervals for the remaining analyses. In contrast to results from the interval analysis, percent mortality among species by habitat type, that is, the effect of sentinel cups placed under heavy vegetation canopy compared with cups placed in open areas, showed substantial variation (Fig. 1). Mean percent mortality was significantly higher ($3.140 \geq t(58) \geq 2.801$; $0.007 \geq P \geq 0.003$) under canopy compared with open areas when data were pooled across the 3 trials for each species, with the exception of *An. dirus* ($t(58) = 1.73$; $P = 0.089$). Throughout this study area, the high canopy of the rubber trees shaded both open and heavy vegetation areas; thus, ultraviolet exposure was not a likely explanation for reduced mortality in open areas. We had expected that the canopy should have protected the sentinel cups from the ULV plume of larvicide and produced lower mortality whereas open areas should have allowed more droplets to reach the cups, producing higher mortality. However, the reverse pattern observed in the data suggests

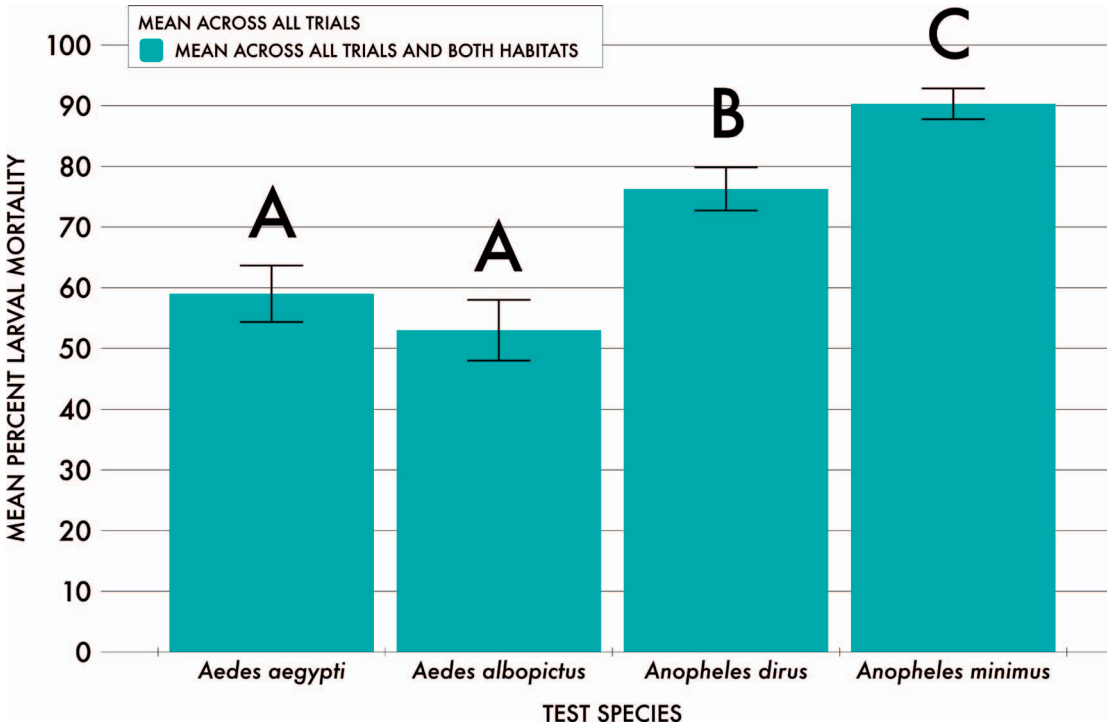

Fig. 2. Mean percent mortality from exposure to spinosad pooled across 3 trials and both habitats among the 4 tested species. A standard error of the mean is indicated on each histogram; mortality significantly varied across species (ANOVA, $F(3, 236) = 17.189$; $P < 0.001$). Letters A, B, and C indicate significantly different mortality ($P < 0.01$) among the 4 species derived from *t*-test pairwise comparisons. A similar analysis of mean percent mortality data by genus pooled across all 3 trials and both habitats indicated significantly greater mortality of *Anopheles* compared with *Aedes* ($t(238) = 6.609$; $P < 0.01$), suggesting greater sensitivity to spinosad in these *Anopheles* laboratory strains. Of the 2 *Anopheles*, *An. minimus* mortality was significantly higher ($t(118) = 3.188$; $P < 0.002$) as shown here.

vegetation canopy could have presented a buffering effect to the wind, trapping and slowing the ULV plume and increasing deposition into the cups. In this model, open areas would hinder the wind less, allowing the ULV plume to move more quickly through the sentinel cup stations with less deposition compared with canopy areas. A related and potentially confounding variable at this study site is that the canopy area was at the foot of a hill whereas the open area was flat, possibly leading to variation in air flow between the 2 segments of the transect. Future investigations will be conducted in more topographically homogenous areas, and additional sentinel cups will be placed at increasing distances from the spray line to distinguish effects of essentially point-blank application into sentinel cups from effects of the ULV plume traveling with air currents through the 2 habitat types.

Pooled percent mortality data across all 3 trials and both habitats by genus indicated significantly greater mortality of *Anopheles* compared with *Aedes* ($t(238) = 6.609$; $P < 0.01$), suggesting less sensitivity to spinosad in these *Aedes* laboratory strains. Reduced sensitivity in *Aedes* is particularly apparent in Trial 1, where mean *Aedes* mortality was approximately 26% and mean *Anopheles* mortality was approximately 72%, a difference of approximately 46% ($t(78) = 11.162$; $P < 0.001$). In contrast, in Trials 2 and 3 the mortality difference between these genera was still significant yet more comparable at approximately 17% ($2.627 \geq t(78) \geq 2.394$; $0.019 \geq P \geq 0.010$). At least among the colony-reared species used in this investigation, the likely low realized flow rate in Trial 1 could have been at a threshold that favored survival of the *Aedes*, and implying that the *Anopheles* species are more sensitive to this larvicide. Of the 2 *Anopheles*, *An. minimus* mortality was significantly higher ($t(118) = 3.188$; $P < 0.002$; Fig. 2) when pooling percent mortality data across trials and habitats. This significant difference was driven by data from Trial 1 where *An. minimus* showed substantially higher mortality than any other tested species even under poor ULV spray conditions. In Trials 2 and 3, mortality in the 2 *Anopheles* species was not significantly different. No significant difference in mortality between both tested *Aedes* species was observed in any trial or grouping of trials.

This investigation shows that Natular 2EC under a combination of field and laboratory conditions shows

promise as an effective long-term residual against 4 medically important mosquito species. This larvicide could be applied as a residual to dry areas that are known to collect water and potentially still be effective after rains or irrigation to enhance protection of humans within a diverse IVM program. This investigation also demonstrates, for the 1st time, efficacy of spinosad against *An. minimus* and *An. dirus*.

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