AMCARF Project Final Report

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Project Title: Effect of size and circadian activity patterns of nontarget pollinators on their chronic and acute susceptibility to adulticides

AMCARF project number: 2020-01

Project Cost: $45,100.00

Project Leader: Lawrence Reeves

Collaborators: Indian River Mosquito Control District

Project Objectives:

- Aim 1. Quantify the effect of size on mosquito adulticide-induced mortality in monarch caterpillars exposed to malathion ULV spray-treated hostplants.
- Aim 2. Determine if malathion-treated host plants infer any protection against parasitism in monarch caterpillars.
- Aim 3. Assess whether patterns of circadian activity and nocturnal resting behavior reduce mosquito adulticide exposure and effects in the monarch butterfly and the common eastern bumblebee.

Total Project Progress:

<u>Key Research Accomplishments:</u>

Figure 1. Monarch colony at FMEL shortly after establishment.

In January 2020, cultivation of tropical milkweed, *Asclepias curassavica*, from seed began at the Florida Medical Entomology Laboratory (FMEL). This milkweed supply was occasionally augmented with additional commercially obtained plants from a supplier known to avoid pesticide use. In April 2020, a self-sustaining monarch butterfly, *Danaus plexippus*, colony (Fig. 1) was established at the FMEL to supply monarch eggs, larvae and adults for the proposed experiments in Aims 1-3. These monarchs were reared in mesh cages inside a screened building using tropical milkweed as a host plant. The colony was founded and occasionally augmented with wild caught adult female monarchs from Vero Beach, Florida. Host plants were provided to caterpillars inside cages by cutting bouquets of milkweed stems and placing them into plastic cups filled with water through the straw hole of their lids. To reduce the incidence of pathogens, particularly nuclear polyhedrosis virus, all milkweed stems were dipped in a dilute sodium hypochlorite (0.074%) solution and thoroughly rinsed and dried before feeding. Host plants were added to caterpillar cages daily or as needed. After caterpillars had consumed all leaves from a bouquet, the cup was moved to a table underneath a grow light to be rooted and ultimately potted and cultivated for later use in caterpillar feeding (Supporting Figure 1). Pupae were transferred to a separate mesh cage. Shortly after eclosion, male and female adults were transferred to a larger mesh cage where they mated. The cage was provisioned with several milkweed bouquets in plastic cups for mated females to lay eggs. Every other day, eggs were transferred to caterpillar rearing cages where they were reared as described above.

Aim 1. We have successfully completed experiments that assessed the effect of size on susceptibility to mosquito adulticides (ULV malathion spray) in monarch caterpillars. We have also extended this aim to include adult butterflies (multiple species), and we have successfully completed an experiment to assess whether butterfly mass affects mortality (described in Aim 3). These experiments (Aims 1-3) were performed in collaboration with the Indian River Mosquito Control District (IRMCD).

The effect of size on susceptibility to malathion ULV spray in monarch caterpillars was assessed by exposing tropical milkweed host plants (*Asclepias curassavica*) to a malathion treatment at two distances from a predetermined spray path, 50 m and 75 m. The spray path in this and all other experiments described below was established along a line perpendicular to the wind direction in an open, mowed field at the Indian River County Fairgrounds in Vero Beach, Florida. At each distance from the spray path, we placed a set of ten potted tropical milkweed plants in a line parallel to the spray path, with a third set of ten plants located upwind of the spray path to serve as a control (Supporting Figure 2). To gauge the effectiveness of the malathion treatment, one screen-enclosed disc cage containing 25 colony-reared adult female *Aedes aegypti* mosquitoes was hung from a shepherd's hook 1 m from the ground at each treatment distance (50 m, 75 m, and control). An IRMCD spray truck then drove the spray route applying malathion via truck mounted ULV spray at a flow rate of 0.005 lbs/acre. Following malathion application, all host plants and mosquito cages were transported back to FMEL, with control host plants transported in a separate vehicle from treated plants to avoid any potential transfer of malathion. Upon arrival at the laboratory, sugar water-soaked cotton strips were placed on top of disc cages to feed mosquitoes, and all mosquito cages were held overnight. Mosquito mortality was assessed after 12 hours. After that time, 100% of mosquitoes located at 50 m and 75 m were dead, while 100% of control mosquitoes were still alive.

Prior to the experiment, a colony of monarch butterflies was established from females collected in Vero Beach, Florida, and maintained on tropical milkweed host plants. Several hours prior to the malathion application, 249 caterpillars of various sizes and instars were selected from the colony. Each caterpillar was individually weighed, its mass was recorded, and the caterpillar was placed in a paper cup with a unique number and its mass written on the side. Once a caterpillar was placed inside the cup, it was closed by affixing a ~7 cm × 7 cm over the top with a rubber band. No host plants were added to the cups. The 249 caterpillar cups were then organized into three groups based on mass: small (<0.07 g), medium (0.07-0.19 g) and large (0.2-0.9 g). Each cup was then haphazardly assigned to a treatment group (50 m, 75 m, or control) such that approximately equal numbers of size classes were assigned to each treatment, and the assigned treatment group was written on the outside of the cup. Following application of malathion to the host plants, the plants were immediately transported back to the lab. Upon arrival, each caterpillar was provided with several host plant leaves from plants of the assigned treatment group (Supporting Figure 3). Over the subsequent five days, each caterpillar was checked daily and fed additional leaves from host plants of the assigned treatment group. At the conclusion of the five days, or if a caterpillar was found dead at the daily checks, each caterpillar was weighed, and its mass was recorded.

We found monarch caterpillars to be surprisingly resilient to malathion ULV spray-treated host plants, and mortality of caterpillars from all size classes and treatments did not exceed 25% (Supporting Figure 4). The mass of small caterpillars from the control group increased most, on average (Fig. 2, Supporting Table 1), during the study period. Small caterpillars fed host plants located at 50 m and 75 m from the spray path also increased in mass over the five-day period, but not to same extent as those from the control group. All caterpillars from the medium size class, including control group caterpillars, lost mass over the five days. Of those from the large size class, individuals fed host plant leaves from the 50 m distance increased in mass more than others. These results suggest that monarch larvae, including the smallest we included (2nd instar) are generally resilient to feeding from host plants that have been treated with malathion ULV spray applications. Interestingly, relative mortality was lowest among the smallest caterpillars that were fed treated host plant leaves, and these larvae increased in mass, indicating that they were able to feed and grow on malathion-treated host plants.

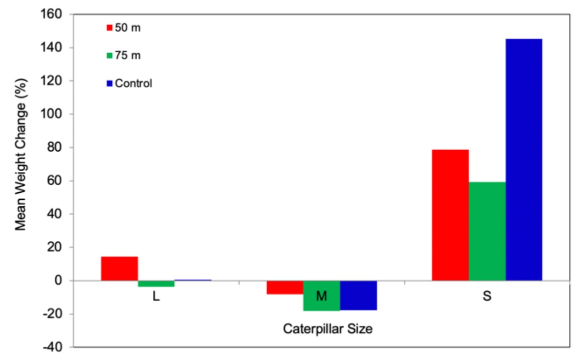

Figure 2. Mean weight change of differently sized of monarch caterpillars, Danaus plexippus *that fed on host plant leaves treated with malathion over 5 days.*

Aim 2. We have successfully completed the planned experiment investigating the potential effects of malathion ULV treatment on parasitism in monarch caterpillars, and have also successfully completed a related experiment investigating the effect of malathion ULV treatment on predation of monarch caterpillars by *Polistes* spp. paper wasps. Initially, we planned to use the tachinid fly *Lespesia archippivora* to assess effects of adulticides on parasitism, but we experienced difficulty in collecting sufficient quantities of wild flies to establish a colony. These difficulties were primarily due to predation of monarch caterpillars by *Polistes* spp. paper wasps in nature. We attempted to collect tachinid parasitoids by placing tropical milkweed host plants with feeding monarch caterpillars in the field in areas where tachinid monarch parasitoids were known to occur. Milkweed plants with feeding caterpillars were placed in plastic trays filled with water to a depth of approximately 2.5 cm to prevent the caterpillars from leaving the plant. Our intent was to leave the plants and caterpillars in the field for several days, then bring caterpillars back into the lab once they reached the 5th larval instar, where they could complete development, and we could collect parasitoids from any field-parasitized caterpillar. However, the vast majority of monarch caterpillars that we placed in the field were attacked and consumed by *Polistes* paper wasps. For this reason, we obtained the commercially available lepidopteran egg parasitoid *Trichogramma platneri*, a species of chalcidoid wasp that parasitizes the eggs of monarchs and other butterfly and moth species. The adult females of these wasps lay their eggs inside lepidopteran eggs (Supporting Figure 5). Their larvae consume the developing lepidopteran embryo, pupate inside the egg, and ultimately, adult wasps emerge from the egg of their host. As a byproduct of these difficulties with *Polistes* predation, we designed and performed another experiment to test the potential for malathion ULV spray-treated host plants to infer protection from *Polistes* predation on monarch larvae.

To assess the effect of malathion treatments on parasitism of monarch eggs, we exposed tropical milkweed host plants to a malathion ULV spray, applied by the IRMCD. As in Aim 1, three sets of three potted milkweed plants were arranged parallel to the spray path (Supporting Figure 6). In this experiment, one set of ten plants was placed in a line 25 m from the spray path, another was placed 75 m from the spray path, and a third was placed upwind from the spray path to serve as a control group. To gauge the effectiveness of the malathion treatment, one screen-enclosed disc cage containing 25 colony-reared adult female *Aedes aegypti* mosquitoes was hung from a shepherd's hook 1 m from the ground at each treatment distance (25 m, 75 m, and control). An IRMCD spray truck then drove the spray route applying malathion via truck mounted ULV spray at a flow rate of 0.007 lbs/acre. Following malathion application, all host plants and mosquito cages were transported back to FMEL, with untreated plants transported in a separate vehicle to avoid any potential transfer of malathion.

Upon arrival at the lab, treated and control host plants were unloaded from vehicles and nine stems were cut from plants of each treatment group (25 m, 75 m, and control). Each stem was approximately 25 cm in length, and included, at the apical end, 10-20 leaves. Each stem was then placed into a plastic cup holding ~75 mL water with a plastic lid. The stem was inserted into the closed plastic cup in place of a straw, with the leaves above the lid of the cup. For three days prior to the malathion application, 10 adult female monarch butterflies were kept inside a mesh cage with access to cut milkweed stems with leaves, upon which they laid eggs. Monarch eggs were removed from these leaves by cutting out the small section (<1 cm^2 in area) of leaf immediately surrounding the egg. The leaf sections with eggs attached were glued to the upper surface of treated or control leaves (Supporting Figure 7) such that each cup containing a treated or control milkweed stem had five monarch eggs affixed to its leaves (N=135). These cups holding the milkweed stems with eggs were then transferred into nine mesh cages (60 × 60 × 60 cm BugDorm Insect Rearing Cages) that each contained approximately 30,000 adult *Trichogramma platneri* wasps (Rincon-Vitova Insectaries Inc.). Each cage was given one cup/stem from each of the treatments (25 m, 75 m) and control. The *Trichogramma platneri* wasps had access to the monarch eggs glued to the treated and control milkweed leaves for 24 hours, and after that time, the cups were removed from the cages. Each egg was then removed and transferred to a Petri dish. The eggs were checked immediately upon removal, and every 24 hours for three days after removal from the parasitoid cages.

Parasitism of monarch eggs was recorded across all treatment groups (25 m, 75 m, control), and ranged from ~65% in the 25 m group to 100% in the control group (Fig. 3). Rates of monarch egg parasitism differed significantly between eggs placed on leaves from the 25 m, 75 m, and control treatment groups (P=0.016). The rate of parasitism of eggs placed on plants nearest to the spray path (25 m) were significantly lower. There was no difference in parasitism rates for eggs placed on

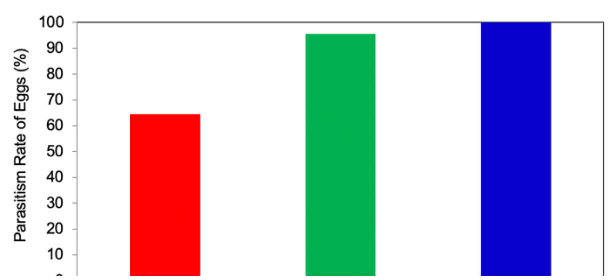

Figure 3. Rate of parasitism by Trichogramma platneri*, on monarch eggs,* Danaus plexippus *affixed to host plant leaves treated with malathion.*

control and 75 m plants, suggesting that there is a benefit inferred upon monarch butterflies by malathion ULV spray applications.

Following our observations of *Polistes* paper wasp predation on monarch caterpillars placed in the field to collect parasitoids we designed an experiment to assess if malathion ULV spray applications to host plants could infer any protection against predation to the monarch larvae. Concurrent with the spray application by IRMCD to treat plants for the parasitoid experiments, we placed an additional 40 potted milkweed host plants in linear arrays parallel to the spray path. Twenty plants were placed at a distance of 25 m from the spray path, and 20 were placed upwind to serve as control plants. Immediately following the application, as described above, the plants were transported back to FMEL and placed outdoors at the Oslo Road Conservation Area, adjacent to FMEL. Each plant was placed into a large bowl that was filled with water to create a moat that would prevent the caterpillars from wandering off the plant. The plants were arranged in the field in ten groups of four plants (Supporting Figure 8). Each group of four plants included one plant that had been exposed to the malathion ULV spray (25 m from the spray path), one control plant that was placed upwind of the spray path, one plant that had been exposed to the malathion ULV spray placed underneath a mesh laundry hamper, and one control plant placed under a mesh laundry hamper. Mesh hampers excluded *Polistes* wasps to monitor for caterpillar mortality due to other causes. We then placed four 3^{rd}, 4^{th}, or 5^{th} instar monarch larvae on each plant (N=160). Caterpillars were checked and mortality was measured at five hours, 21 hours and 25 hours after the start of the experiment.

Interestingly, we found that monarch larvae feeding from treated plants were significantly more likely to have been removed from their host plant after 25 hours ($P<0.0001$). None of the caterpillars on host plants protected under mesh laundry hampers were missing. After 25 hours, 70% of caterpillars on treated plants had been removed, presumably by *Polistes* paper wasps, while 30% of caterpillars from control plants had been removed. It is possible that feeding on treated host plants changes the feeding or defensive behavior of monarch larvae, making them more apparent or susceptible to attacks by predators. This suggests that there is not a benefit inferred upon monarch larvae feeding from malathion-treated host plants that reduces predation from these comparatively large-bodied predators.

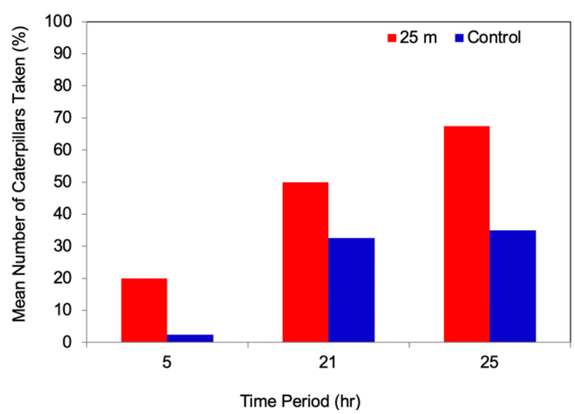

Figure 4. Rate of predation by paper wasps, Polistes *sp. on monarch caterpillars,* Danaus plexippus *feeding on host plants treated with malathion. Mean number of caterpillars killed or taken is shown.*

Aim 3. We have successfully completed experiments to assess whether patterns of circadian activity and nocturnal resting behavior reduce mosquito adulticide exposure and mortality in the monarch butterfly and the common eastern bumblebee. One challenge we experienced that resulted in changes to our proposed procedure was that we did not have a sufficiently large number of adult monarch butterflies at the time of our malathion treatment. To overcome this, we augmented the number of adult monarchs with additional wild-collected individuals of multiple butterfly species (*Agraulis vanillae*, *Heliconius charithonia*, *Anartia jatrophae*, *Phoebis sennae*, *Papilio glaucus*, *Papilio palamedes*, *Ascia monuste*, *Junonia coenia*, *Limenitis archippus*). This adjustment was beneficial not only to increasing the sample size, but allowed a wider range of butterfly size/mass classes to be included in the experiment, enabling us to glean information on the effect of mass on susceptibility to malathion ULV application.

We designed an experiment to examine the effect of nocturnal roosting behavior (roosting height and roosting amongst vegetation) on mortality due to mosquito adulticide exposure. To investigate the effect of nocturnal roosting behavior on mortality due to exposure to mosquito adulticide ULV spray treatments, we constructed three seven-meter tall bamboo towers and placed butterflies at three heights on each tower. The towers were located at 25 m and 75 m from a predetermined spray path, and one tower was located upwind from the spray path to serve as a control (Supporting Figure 9). On each tower, approximately 20 butterflies were affixed to strings with plastic clothes pins (Supporting Figure 10) at three heights from the ground: 1 m, 4 m, and 7 m. Approximately 60 butterflies were pinned to strings on each tower (N=184). Each butterfly was given a unique identifying number, written on one of the hindwings with a black Sharpie marker, and the mass of each butterfly was recorded prior to the start of the experiment. At each height on the tower (1 m, 4 m, 7 m), six butterflies were artificially protected to mimic nocturnal refugia. For these butterflies, a sprig of saltbush, *Baccharis halimifolia*, consisting of a branch with leaves was placed in front of the butterfly to mimic the nocturnal roosting habits of butterflies. Disc cages each containing 25 lab-reared adult female *Aedes aegypti* were placed at each height on each tower alongside the butterflies to ensure that the treatment was successful. Once all towers with butterflies and mosquitoes were in place, an IRMCD spray truck drove the spray route applying malathion via truck mounted ULV spray at a flow rate of 0.007 lbs/acre. The towers were then dismantled, and the butterflies were placed into mesh cages for transport back to the lab. One mesh cage was used to hold all butterflies from each tower height and distance from spray path to ensure no transfer of residual malathion between treatment groups. The butterflies were held in the cages overnight and fed with dilute Gatorade (applied to folded paper towels placed on top of the mesh cages). Mosquitoes were held overnight in the disc cages and were given sugar water applied to cotton strips placed on top of the cages. Butterfly and mosquito mortality were assessed after 12 hours.

Adult butterflies were relatively resilient to malathion ULV spray application (Supporting Table 2), though there was significant variation in mortality between groups located at various heights from the ground and distances from the spray path (Fig. 5). Mortality between 25 m, 75 m, and control groups was significantly different between all groups ($P<0.0001$). Similarly, height significantly affected mortality ($P<0.0001$). There was no difference in mortality between 1 m and 4 m heights, but mortality at 7 m differed significantly from both 1 m and 4 m heights in at the closest distance from the spray route (25m; $P=0.0001$). The majority of butterflies (~85%) located at 7 m were still alive and appeared healthy 12 hours across all treatments. The presence of a vegetation refugium did not affect survival of butterflies by treatments; 25 m ($P=0.8951$), 50 m ($P=0.8753$), and Control ($P=0.2634$). Mortality was not affected by the mass of the butterfly ($P=0.82$; Supporting Figure 11).

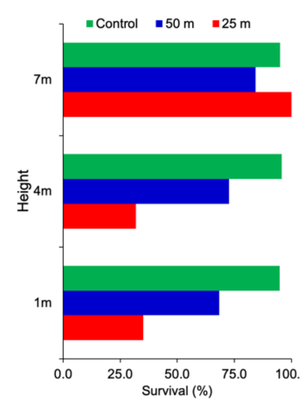

Figure 5. Effect of malathion on adult butterflies of various species at different roosting sites and distances from adulticide spray route.

To assess the effect of nocturnal behaviors on susceptibility to mosquito adulticide applications on bumblebees commercially obtained bee, *Bombus impatiens* (Koppert Biological Systems Inc.) were exposed to a malathion ULV application. Bumblebees with and without (Supporting Figure 12) a nocturnal refugium (hive) were positioned 50 m from a predetermined spray path. Four bumblebee hives were placed along a line parallel to the spray path such that each was a distance of 50 m from the spray. The main entrance/exit to the hive was closed, but the hive still had some perforations that allowed for air flow. Next to each hive, we placed mesh cages into which we released approximately 20 individual workers from the corresponding hive. Two bumblebee hives and cages were set up identically upwind of the spray path to serve as controls. Once all hives and cages were in place, an IRMCD spray truck drove the spray route applying malathion via truck mounted ULV spray at a flow rate of 0.005 lbs/acre. Following application, all hives and wire-mesh cages were transported back to FMEL, held overnight, and mortality was measured 12 hours later. Although observed survival was reduced in bumblebees that were exposed to malathion ULV spray, bumblebees were generally resilient, even those fully exposed to the application. Overall, 87% of exposed bumblebees were alive and behaving naturally 12 hours after application (Supporting Figure 13, Supporting Table 3). Comparatively, 97% of bumblebees within hive refugia were alive, however the difference was not significant. Mosquitoes in disc cages located alongside the bumblebees at 50 m from the spray were all (100%) killed by the application, whereas all mosquitoes located upwind of the treatment were alive 12 hours later.

Reportable Outcomes:

No papers, inventions filed, or patents issued to date.

Our project has advanced the field of mosquito control scientifically by providing data on the nontarget impacts of mosquito adulticide applications on butterflies and bumblebees, two high profile pollinator groups. The majority of nontarget impact research has focused on a limited range of nontarget pollinator taxa, in particular the European honeybee, and on direct, exposure-related effects. Our project focused on how natural behaviors of butterflies and bees may limit their susceptibility to adulticide applications (e.g., roosting among vegetation, roosting above ground level, nocturnal resting within a hive), and on potential benefits of mosquito adulticide applications to butterflies (e.g., reduced parasitism, reduced predation). These data are valuable to improving understanding of the real-world nontarget impacts of adulticide applications, and will enable mosquito control districts to better limit nontarget effects of adulticide treatments, as well as helping to address concerns related to potential nontarget effects.

We are currently finalizing statistical analyses associated with **Aims 1-3**, and we plan for these results to be developed into a scientific publication to be submitted in 2021.

Progress Assessment:

- Aim 1. We have successfully completed the proposed experiment assessing the impact of size on the susceptibility of monarch caterpillars to malathion ULV spray-treated host plants. We have augmented this aim with an assessment of the effect of size on the susceptibility of adult butterflies to malathion exposure. Statistical analyses of the results from these assessments are currently being finalized and will be presented in a forthcoming publication along with results from Aims 2 and 3.

- Aim 2. We have successfully completed the proposed experiment assessing if malathion-treated host plants infer any protection against parasitism in monarch caterpillars. We had a challenge with this Aim in that we experience difficulty in collecting the planned species of parasitoids. Rather than using the tachinid fly *Lespesia archippivora*, a parasitoid of monarch caterpillars, we used the commercially available hymenopteran parasitoid *Trichogramma platneri*, a parasitoid of monarch eggs. We also augmented this Aim by performing an additional experiment assessing if malathion ULV spray applications infer any protection against predation by *Polistes* paper wasps. Statistical analyses of the results from these assessments are currently being finalized and will be presented in a forthcoming publication along with results from Aims 1 and 3.

- Aim 3. We have successfully completed the proposed experiments assessing whether patterns of circadian activity and nocturnal resting behavior reduce mosquito adulticide exposure and effects in the monarch butterfly and the common eastern bumblebee. We investigated whether the nocturnal behaviors of bumblebees retreating to hives at night, and butterflies roosting among vegetation impact their susceptibility to malathion ULV spray applications. For butterflies, we added an additional variable, roosting height, into the experiment, and measured differences in susceptibility for butterflies roosting at 1 m, 4 m, and 7 m from the ground. Statistical analyses of the results from these assessments are currently being finalized and will be presented in a forthcoming publication along with results from Aims 1 and 2.

  Green = successfully completed

Plans for the following year: All experiments and data collection are complete. Statistical analyses of the results are in the process of being finalized, and we expect to present these at the 2021 Annual AMCA Meeting, and in a forthcoming publication in 2021.

Conclusion: AMCARF project number: 2020-01 “Effect of size and circadian activity patterns of nontarget pollinators on their chronic and acute susceptibility to adulticides” represents an investigation of several factors that may affect the susceptibility of nontarget pollinators, butterflies and bumblebees, to mosquito adulticide applications, and of potential benefits to nontarget butterflies via reductions in the impacts of their natural enemies. We completed all proposed experiments, assessing the impacts of caterpillar size (Aim 1) and nocturnal resting behaviors (Aim 3) on susceptibility to mosquito adulticides, and of mosquito adulticides applications on parasitism of monarch eggs (Aim 2). We were able to augment the proposed experiments with experiments investigating the impacts of mosquito adulticide application on predation of monarch caterpillars by paper wasps, and the impact of size on susceptibility to mosquito adulticides in adult butterflies. We also added an additional variable, roosting height, to our experiment assessing nocturnal roosting behaviors of butterflies and susceptibility to mosquito adulticides.