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Semi-field evaluation of a novel controlled release device using transfluthrin as spatial repellent to prevent entry of mosquitoes into military tents

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ABSTRACT

Mosquitoes can impact military operational readiness by transmission of disease-causing pathogens or through secondary effects, e.g., annoyance and bites. The focus of this research was to determine if an array of novel controlled release passive devices (CRPD) utilizing the spatial repellent, transfluthrin (TF), as the active ingredient could prevent entry of mosquitoes into military tents for up to 4 weeks. The TF-charged CRPDs were spaced along six strands of monofilament and hung across the tent entrance. Efficacy was evaluated with caged *Aedes aegypti* to indicate knockdown/mortality effects, and four species of free-flying mosquitoes, *Ae. aegypti*, *Aedes taeniorhynchus*, *Anopheles quadrimaculatus* and *Culex quinquefasciatus*, to indicate repellent effects. Bioassay cages containing *Ae. aegypti* were hung vertically at 0.5, 1.0 and 1.5 m above ground level at designated locations inside of the tents. Knockdown/mortality counts were made every 15 min for the first hour, then at 2, 4 and 24 h post-exposure. Free fliers were recaptured in BG traps operated from 4 to 24 h post-exposure. Knockdown/mortality was gradual until 4 h post-exposure. This increased to near 100% by 24 h in the treated tent but was < 2% in the control tent. There was a significant reduction in the recapture rates of all free-flying species in the treated tent compared with the control tent. Results indicate that TF-charged CRPDs can significantly reduce the numbers of mosquitoes entering military tents and that the four species were affected similarly by the TF. The needs for additional research are discussed.

1. Introduction

Arthropod-borne diseases, such as malaria, dengue, scrub typhus and leishmaniasis, continue to pose a significant threat to deployed U.S. military forces. Biting arthropods not only transmit disease (Riddle et al., 2008), but as persistent pests they can inflict painful and distracting bites that may lead to secondary infections, dermatitis, or allergic reactions (Kitchen et al., 2009). Traditional methods used to minimize exposure include application of residual insecticides on tents and buildings: use of barrier sprays, ultralow volume (ULV) or thermal fogging applications of insecticides and use of personal protective measures (PPM), such as the application of topical insect repellent on exposed skin, wearing permethrin-treated uniforms, and the use of insecticide-treated bednets (Maroli & Khoury, 2004; Coleman et al., 2006).

In instances of limited impact from these methods, failure has been attributed to unavailability, non-compliance, improper use, and ineffectiveness of some of the products (Coleman et al., 2011). Coleman et al. (2011) suggested that new technologies were needed to protect the deployed soldiers. The use of area-wide or spatial repellents has been suggested as a possible alternative based largely on studies reported by Ogoma et al. (2012b). Their studies demonstrated the potential of spatial repellents to achieve long-term area protection of humans from mosquito bites (Ogoma et al., 2012b, 2017). In these studies, effective protection was achieved by maintaining adequate levels of the active ingredient, transfluthrin (TF), in the air.

It was determined (Lloyd et al., 2013) that spatial repellent devices using insecticides were the most efficient at providing protection but none of the commercially available devices was ideal for use during deployments, highlighting a need for the development of a military-grade spatial repellent device. Lloyd et al. (2013) suggested that the ideal device should be versatile (indoor/outdoor), portable, tactical, easily deployable, and contain repellent insecticides that vaporize at ambient

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temperatures.

Thus, the objective of our research was to evaluate a novel controlled release passive device (CRPD) developed in our laboratory using TF spatial repellent as the active ingredient. The device should remain active for 4 weeks. CRPDs require minimal involvement of the deployed soldier with the goal of minimizing non-compliance issues associated with use of topical repellents. The CRPDs are designed for placement in an array at a tent entrance to prevent mosquito entry and minimize vector-soldier contact.

2. Materials and methods

2.1. Semi-field study site

Semi-field studies were conducted at the USDA-ARS-CMAVE, Gainesville, FL, USA, in two outdoor screened enclosures (each 9.1 × 18.3 × 4.9 m high, pitched to 5.5 m with metal frames). The long axis of the enclosures is oriented north to south, and the enclosures are parallel to each other and 24 m apart. An entry door is on the southeast corner of each enclosure. Each enclosure contained a military tent (HDT Base X Model 305 Shelter, HDT Global, Solon, OH, USA) with a floor space of 5.5 × 7.6 m long, walls 2.24 m high and the roof pitched to 3.1 m high at the peak (Fig. 1). Tent was in the south end of the enclosures and tent openings were 1.22 × 1.91 m high and faced north. The treated tent was in the west enclosure and the control tent was in the east enclosure.

2.2. Mosquitoes and bioassay cages

Mosquito strains used in these studies were from CMAVE colonies and included pyrethroid-susceptible Orlando strains of *Aedes aegypti*, *Aedes taeniorhynchus* and *Anopheles quadrimaculatus* (all maintained since 1952), and a Gainesville 1996 pyrethroid-susceptible strain of *Culex quinquefasciatus* (Allan et al., 2005). All were maintained in the CMAVE insectaries using previously published procedures (Gerberg et al., 1994; Allan et al., 2005). Colonies were kept at room temperature (22.5 °C) or in an incubator (27 °C) with a photoperiod of 14:10 (L:D) h and *ad libitum* access to 10% sucrose-soaked cotton.

When caged mosquitoes were used to determine knockdown/mortality effects, they were housed in bioassay cages. These were made from cardboard rings (Multi Packaging Solutions, Chicago, IL, USA), and consisted of one wide inner ring (15.2 cm in diameter × 3.8 cm wide) and two narrower but slightly larger in diameter outer rings (15.9 cm in diameter × 1.6 cm wide) into which the inner ring would fit. A circle of tulle fabric cut slightly larger than the diameter of the wide inner ring was laid and centered across a wide ring placed on its side. An outer ring was slipped over the tulle and onto the inner ring holding the tulle tightly in place. The process was repeated for the opposite side of the inner ring. A 1.3-cm hole centered in the rim of the wide inner ring was used for placement of dental wicks or cotton saturated with sucrose solution as needed. The tulle fabric can be easily removed to put mosquitoes inside the cage. Twenty-five 5- to 6-day-old female *Ae. aegypti* mosquitoes were put into each cage after being knocked down in a cold room at 5 °C degrees for 10 min. Mosquitoes were allowed to fully recover before the bioassay cages were used in the tent studies.

In the first study increment to determine how free-flying mosquitoes would respond to the TF, 300 *Ae. aegypti* 5- to 8-day-old females were released in the screened enclosures containing the treated and untreated tents after the 4-h knockdown count (see below). In study increments 2–4 to determine if different mosquito species would respond differently to the TF, 5- to 8-day-old female mosquitoes, 300 each of *Ae. aegypti*, *Ae. taeniorhynchus*, *An. quadrimaculatus* and *Cx. quinquefasciatus*, were released after the 4-h knockdown count (see below). Mosquitoes were released simultaneously in the opposite end of the screened enclosures from where the tents were placed.

2.3. Controlled release passive devices (CRPDs)

The CRPDs utilized in this study are multi-lumen devices constructed from different sizes of polypropylene drinking straws. The outer straw (Comfy Package, Brooklyn, NY, USA) has an 8-mm-diam. lumen which encloses two smaller straws (KCH Corporation, Brooklyn, NY, USA), each with 3-mm-diam. lumens. Cotton (0.5 g) was packed in the 8-mm lumen in the space not occupied by the two smaller 3-mm straws to contain and release the spatial repellent formulation (Fig. 2). The CRPDs are 2.5-cm long and open at both the ends. CRPDs were attached to monofilament fishing line for ease of spacing and placement.

CRPDs were activated by saturating the cotton with 0.75 ml of 30% TF dissolved w/w in benzyl alcohol. The mixture of TF and benzyl alcohol form an azeotrope-like mixture and have the same composition in the vapor state as in the liquid state.

2.4. Experimental set-up and design

The TF-activated CRPDs were suspended in 6 parallel vertical rows at

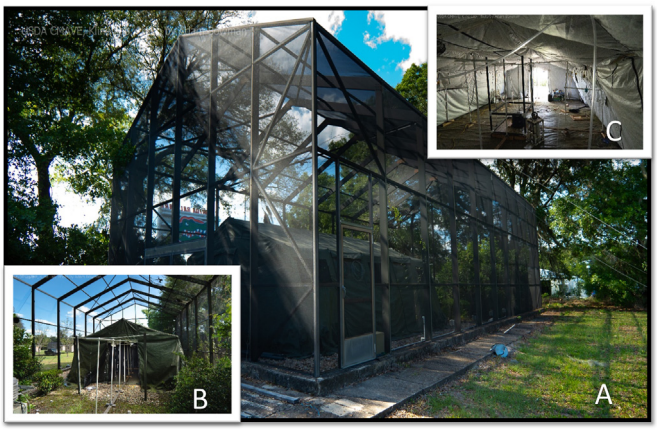

Fig. 1. The outside view of the semi field screened enclosure housing (**A**) the treated military tent (**B**) a view of the tent entrance, and (**C**) an inside view of the tent showing the structure used to suspend the bioassay cages.

Fig. 2. Treated tent entrance showing controlled release passive devices (CRPDs) spanning the entrance. The inset photos show a close-up of a CRPD from a top-down and side view. Lower far right image is a CRPD schematic.

the top margin of the treated tent entrance. The rows, spaced 20 cm apart, were comprised of 10 CRPDs spaced 10 cm apart on monofilament fishing line (Fig. 2). Rows of non-activated CRPDs were not placed at the entrance of the control tent because preliminary studies showed no change in mosquito entry into the tent with or without the presence of non-activated CRPDs.

Mosquitoes in bioassay cages were used to determine knockdown/mortality effects produced by the rows of CRPDs at the tent entrance. To enable placement of bioassay cages in a more-or-less 3-dimensional array inside the tents, a framework was made from ¾-inch polyvinyl chloride (PVC) pipe. Starting at the tent door, 7 vertical posts fastened onto 2- × 4-in lumber were placed along the longitudinal center of the tent floor. Posts were spaced 1 m apart and the distance from post 7 to the rear wall of the tent was 1.5 m. A row of 6 posts was placed midway between and perpendicular to posts 4 and 5 with 3 posts on each side of the longitudinal row, all spaced 1 m apart. Horizontal lengths of PVC connected near the tops of the posts provided stability. Near the top of each post a pair of 43-cm PVC pipes in opposite sides of a tee connector was mounted perpendicular to the long axis of the row of posts. These formed T's and created stations from which to suspend the bioassay cages. By means of wiring in the roof of the tent, four additional remote stations were created on each side of the longitudinal row of posts. Remote stations were adjacent to and 1.5-m from posts 2, 3, 6 and 7. The 1-m^2 area between longitudinal posts 4 and 5 and perpendicular posts 3 and 4 was framed at ground level with 2 × 4-in lumber which was then covered with a fitted piece of 0.5-in plywood to form a central platform.

In the treated tent, bioassay cages were suspended vertically with flexible 18-gauge steel wire (Hillman Group Inc., Cincinnati, OH, USA) from 15 stations (shown in Fig. 3), with 3 cages suspended on the same wire at 0.5, 1.0 and 1.5 m above ground at each station. These heights were equivalent to the height of DLK's knees, waist, and shoulders, respectively. In the control tent two bioassay cages were hung at the tent entrance on the left and right, two in the rear on the left and right of post 6, and one over the central platform. Control cages were hung vertically only at the 1.5-m level.

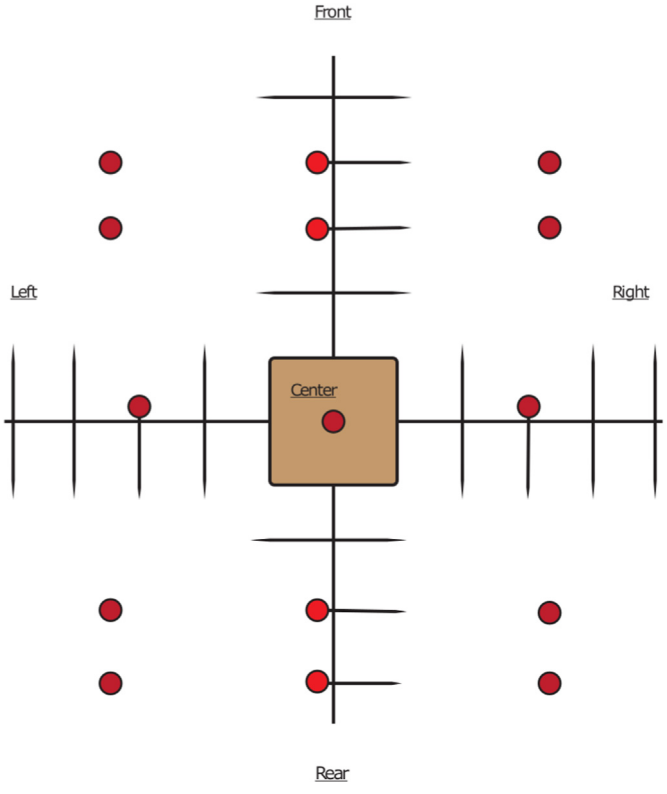

Fig. 3. The entrance to rear groupings of the bioassay traps used in statistical analysis of *Aedes aegypti* knockdown counts. Left to right side groupings were not significant and are not shown. Each red dot represents a vertical grouping of 3 bioassay traps.

If there was a concentration of TF at any location within the tent sufficient to cause knockdown of caged mosquitoes, then it was assumed that the level of TF should be detected by the free-flying mosquitoes and cause them to be repelled. Results from bioassay cages would thus provide an indication of the dispersion pattern of the TF. Because the main purpose of the caged insects was to determine knockdown effects, only one species, *Ae. aegypti*, was used in the bioassay cages. Knockdown was defined as when the mosquitoes are incapable of flight. Counts in control and treated tents were compared.

To monitor the potential of the CRPDs at the tent entrance to prevent free-flying mosquitoes from entering the tent, a BG-Sentinel trap (Biogents AG, Regensburg, Germany) was placed on the central platform described above in each tent. The trap was baited with CO_2 and a BG-Lure ® (a human odor mimic consisting of lactic acid, fatty acids, and ammonia). The CO_2 was delivered near the trap entrance with PVC tubing connected to a 9-kg compressed gas cylinder utilizing Clarke's (Clarke, St Charles, IL, USA) FLOWSWT1. This consists of a regulator (REG1) with a fixed output of 15 psi, with an in-line flow restrictor (ORIF7) and a 10 Micron filter (FILT1) which provides a steady CO_2 flow of 500 ml/min. The BG-Lure, effective for 5 months, was placed in the designated hole in the trap's lid.

2.5. Meteorological conditions

During the period of study, ambient weather conditions which included temperature, humidity, and wind speed were recorded every 30 s continuously for each 24-h period of testing using a Kestrel 4500 NV pocket weather tracker (Boothwyn, PA, USA).

2.6. Study initiation and completion

This study was conducted once a week in for 4 weeks from 8 to 29 June 2021. After the TF-activated CRPDs were suspended at the entrance of the treated tent to begin the study they remained in place until the end of 4th study increment. No additional TF was used. Weekly study increments were designed to evaluate the efficacy of the TF-activated CRPDs over time. To begin the study, bioassay cages with mosquitoes were suspended from the 15 selected stations inside the treated tent and the 5 selected stations inside the control tent. The TF-activated CRPDs were suspended at the entrance of the treated tent and knockdown timing began. The numbers of mosquitoes knocked down in the bioassay cages were recorded every 15 min for the first hour, and then at 2, 4 and 24 h post-exposure. After the 4-h knockdown count the BG-Sentinel trap in each tent was switched on and the free-flying mosquitoes were released from the north end of both screened enclosures. Each study increment was terminated after the 24-h knockdown count and mosquitoes captured in the BG-Sentinel traps were collected and stored in a freezer to be counted and identified to species.

2.7. Statistical analysis

All statistical analyses were performed using R v 4.0.3. *Tidyverse* package v. 1.3.1, and R *stats* package v 4.0.3. An array of 45 bioassay cages ($n = 25$ mosquitoes per bioassay cage) from the treated tent were used in this analysis. The data were modeled in a way to show knockdown rates over 7 time points up to 24 h.

For analysis, groups of bioassay cages were blocked from the front to the rear of the tent and from the left to the right (Fig. 3). This allowed for the analysis of knockdown counts laterally in the tent, and from the entrance to the rear over time. Analysis was also performed without the blocks to show knockdown counts over time using the entire tent. These data did not pass testing for normality and were unable to be transformed

to fit a normal curve. A non-parametric Kruskal-Wallis model was used to compare knockdown rates *vs* time and location. Dunn's multiple comparison was used for *post-hoc* testing for any significant results from the Kruskal-Wallis test.

Knockdown counts in the array of 5 bioassay cages hung in the control tent were conducted in parallel during each replication of the study. The controls were limited to cover the length and width of the tent to determine if any other environmental factors were responsible for mosquito mortalities or knockdowns. Placement of a full array of 45 bioassay cages in the control tent was not necessary because little or no knockdown of controls was observed in preliminary studies.

3. Results

The effects of the TF-activated CRPDs to *Ae. aegypti* mosquitoes in bioassay cages were similar during the entire 4-week study. The exception was day 1 of increment 1 where it appeared that TF was released in greater-than-expected amounts from the recently activated CRPDs. This first increment had significantly different knockdown counts over time when comparing with the 2nd, 3rd, and 4th iterations (Dunn's multiple comparison test, $P < 0.001$). There was an 80–100% knockdown of mosquitoes in all bioassay cages in the treated tent when the 1-h counts were recorded in increment 1. In increment 4 there was an unexpected rapid increase in knockdown between the 2-h and 4-h counts that was not as pronounced for increments 2 and 3. We have no explanation for this effect (Fig. 4).

For the first and second increments, the knockdown counts were significantly higher in the blocks of traps at the front of the tent than in the rear ($\chi^2 = 19.94$, $df = 2$, $P < 0.001$ and $\chi^2 = 9.23$, $df = 2$, $P \leq 0.001$, respectively). There were no front to rear significant differences in knockdown counts in the third and fourth increments, and no significance differences between left to right blocks in increments 1–4. Between the 4-h and 24-h counts knockdown increased throughout the treated tent to essentially 100% in all bioassay cages in all 4 increments. Because of the experimental design, i.e. the long interval between the 4-h and 24-h counts, the actual times required to reach the knockdown/morality levels counted at 24 h, usually close to 100%, remain unknown.

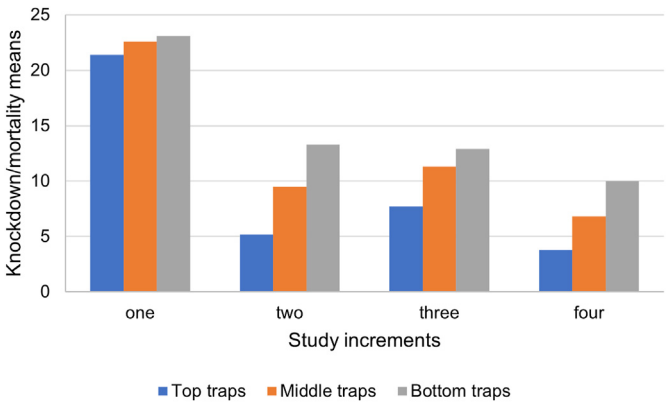

Fig. 5. Knockdown/mortality of *Aedes aegypti* in top, middle and bottom bioassay cages in study increments 1–4.

Knocked down mosquitoes sometimes regained flight and were not counted as knocked down in subsequent observations. Thus, some knockdown/mortality values recorded later in time from the same bioassay cages were slightly lower than earlier ones. Occasionally mosquitoes regained flight after knockdown occurred, but at the 24-h count a majority of the knocked down mosquitoes were either dead or incapable of flight. In the control tent there were zero to two knockdowns in all bioassay cages from the start to the completion of each 24-h study increment.

Knockdown/mortality means in the bioassay cages suspended at three different levels were always in the following order for the four study increments: bottom cages > middle cages > top cages (Fig. 5). In study increment 1 there were no differences in knockdown/mortality means due to cage height. In the study increments 2–4, the bottom bioassay cages had significantly greater knockdown means than the top cages ($Z = 5.051875$, $P < 0.0001$), and the middle cages ($Z = 2.230515$, $P = 0.0257$). The middle cages had significantly greater knockdown means than the top cages ($Z = 2.821360$, $P = 0.0096$).

Mean recapture counts of free-flying mosquitoes released after the 4-h

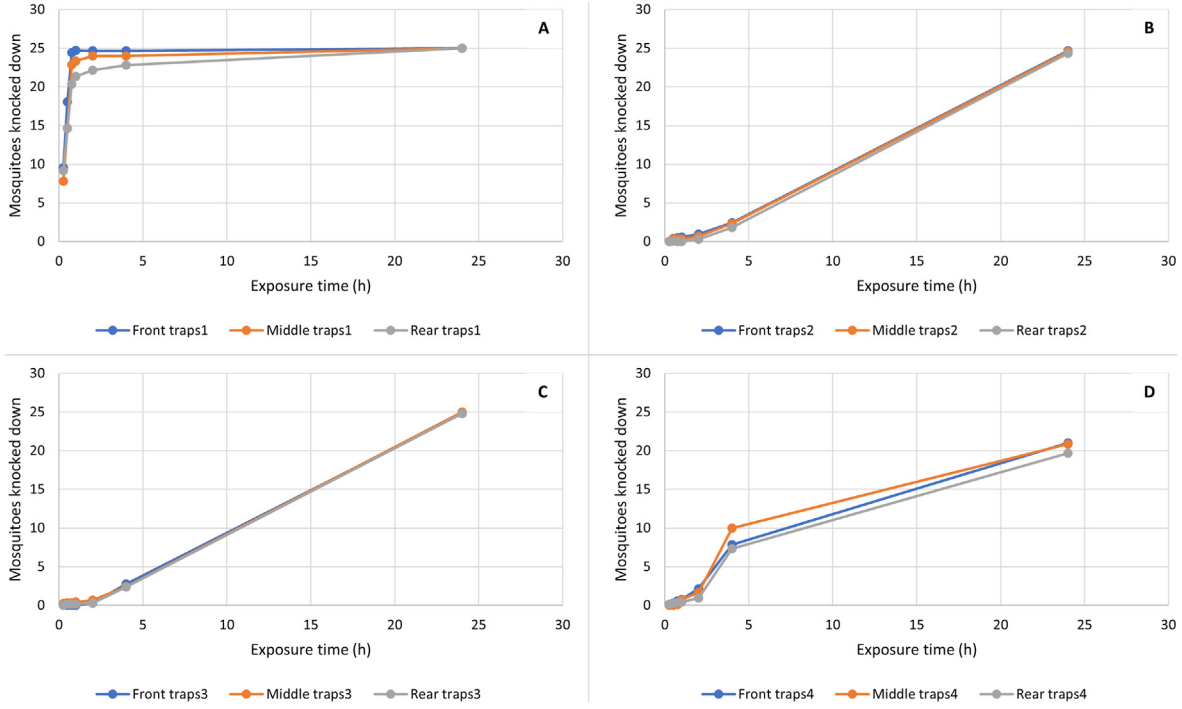

Fig. 4. Knockdown/mortality curves for *Aedes aegypti* mosquitoes in bioassay cages during the 24-h periods in study increments 1 (**A**), 2 (**B**), 3 (**C**) and 4 (**D**).

knockdown count and recaptured after the 24-h count were significantly lower for all species in the treated tent compared with those in the control tent ($F_{(1,18)} = 753.37$, $P < 0.001$). There were no significant differences in recapture means of free-flying mosquitoes among increment dates ($F_{(3,18)} = 0.698$, $P = 0.565$).

Mean recapture counts of free-flying mosquitoes in the treated tent were not significantly different among the mosquito species. Percentages recaptured were: 14.7% (*Ae. aegypti*); 6.89% (*An. quadrimaculatus*); 5.22% (*Cx. quinquefasciatus*); and 1.67% (*Ae. taeniorhynchus*). These percentages are based on the mean recaptures of 300 of each species released on all 3 increment dates (in the first study increment only 300 *Ae. aegypti* were released).

Mean recapture counts of free-flying mosquitoes in the control tent were significantly different among species ($F_{(3,18)} = 39.84$, $P < 0.001$). *Aedes taeniorhynchus* recaptures were significantly lower than the other 3 species (Tukey's HSD test, $P < 0.001$). The average control tent recapture rates for study increments 2–4 were: 79.2% (*An. quadrimaculatus*); 77.4% (*Ae. aegypti*); 75.2% (*Cx. quinquefasciatus*); and 42.7% (*Ae. taeniorhynchus*).

There were some expected statistical differences in temperature and humidity across study dates, but there was no significant correlation (Kendall's rank correlation *tau*) between knockdown counts and temperature ($Z = 0.82527$, $P = 0.4092$, $tau = 0.0171$) or humidity ($Z = 0.79125$, $P = 0.4288$, $tau = 0.0170$).

4. Discussion

The objectives of this study were to develop a CRPD for use with TF and which repelled mosquitoes for up to four weeks. This was essentially accomplished. The CRPDs hung at the entrance of the treated tent were not serviced in any way during the 4-week study. In a short (1 hour/replication) proof-of-concept study, McPhatter et al. (2017) showed that TF applied to surfaces reduced the numbers of mosquitoes entering small tents. References to various spatial repellent devices can be found in the literature (Pates et al., 2002; Ogoma et al., 2012a); however, emphasis in our work was on the development of a passive device that allowed the repellent to volatilize into the environment. Although TF-treated hessian cloth is at times referred to as a passive device, our CRPD is a small self-contained unit that can be easily transported, activated, and deployed. Many studies with hessian cloth were conducted outdoors to protect nearby human subjects (Masalu et al., 2017, 2020; Ogoma et al., 2017) and hessian cloth remained active in the field for 1 year (Ogoma et al., 2017). The CRPD is newly developed, and the first semi-field studies are reported herein. Additional evaluations will be necessary to determine how the qualities of the CRPD compare with those of treated hessian cloth.

There was a rapid knockdown/mortality of mosquitoes in the bioassay cages during the first hour of the first increment of the study. The CRPDs had just been activated and hung in the treated tent and a large amount of TF was apparently released into the air. No such effect was observed in the three remaining study increments. Apparently sometime between the end of increment 1 and the beginning of increment 2 the TF release rate became more uniform, as suggested by Jiang et al. (2019).

The knockdown/mortality patterns over time for mosquitoes in bioassay cages were similar during the three remaining increments. A slight unexplained difference occurred during increment 4 that caused knockdown/mortality to increase rapidly between the 2-h and 4-h counts; and knockdown/mortality did not reach 100%. These similar patterns reflect the uniform TF evaporation rate reported by Jiang et al. (2019). This pattern also strongly indicates that exposure time was required to cause increases in knockdown/mortality. Martin et al. (2020) recorded significant differences in knockdown/mortality resulting from the distance the caged mosquitoes were placed from the TF source. Despite the fact that TF was released continuously by the CRPDs during our study, the TF apparently never accumulated in the treated tent in levels high enough to greatly change the patterns of knockdown/mortality over the same exposure time.

The knockdown/mortality numbers over time for mosquitoes in bioassay cages decreased with cage elevation above the floor. Means were tightly grouped in study increment 1 but were more separated in study increments 2–4 (Fig. 5). Martin et al. (2020) also found increased knockdown/mortality closer to the floor. This was expected because TF is a heavier-than-air molecule (Jiang et al., 2019).

TF from the CRPDs reduced the numbers of mosquitoes entering the treated tent by 85–98% when vertical rows of CRPDs were spaced 20 cm apart. TF-treated hessian cloth eave ribbons fitted to eaves of huts reduced the numbers of mosquitoes entering huts through 5-cm eave openings by > 99% (Mmbando et al., 2018). This compares favorably with our results. The species recaptured in the lowest numbers in the treated and untreated tents was *Ae. taeniorhynchus*. Martin et al. (2020) found that two mosquito species in their study reacted differently to TF. However, in our study it seems that *Ae. taeniorhynchus* might be more reluctant to enter the tents than the other species.

More research is needed to further evaluate various effects of the CRPDs. However as expected (Kline et al., 2021), the TF created a protected space where mosquito numbers were greatly reduced and minimized or eliminated the non-compliance issues associated with application of topical repellents (Norris & Coats, 2017).

5. Conclusions

This semi-field study has demonstrated the efficacy of the novel controlled release passive devices (CRPDs) using transfluthrin (TF). The knockdown rates of the caged mosquitoes were faster in the first 1-week study increment and were more uniform over subsequent study increments. Almost 100% knockdown of caged mosquitoes was achieved in 24 hours in all four study increments. The knockdowns were significantly faster and higher in the front of the tent closer to the CRPDs when compared to the rear end of the tent. This can be attributed to the fact that transfluthrin is a heavier-than-air molecule and requires time to permeate all the way to the rear end of the tent. The recapture of free flying mosquitoes in the treated tent for all the four species shows an average repellency rate of 80% when compared to the control tent. This is evident from the data collected from the traps over the four weeks of testing. The mosquitoes which entered the tent and captured in the tent were also completely knocked down. This is an added advantage as the mosquitoes that evade repellency and enter the tent are also rendered inactive, thereby preventing biting. The slow release of TF from the solution enables the CRPDs to remain active and efficient.

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Ethical approval

No ethical approval was required for this study.

CRediT author statement

Nagarajan R. Rajagopal: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Validation, Writing – original draft, Writing – review & editing, Visualization. Adam R. Bowman: Formal analysis, Investigation, Resources, Writing – original draft, Visualization. Floyd J. Aldana: Investigation, Resources. Christopher D. Batich:Writing – original draft, Writing – review & editing, Supervision. Jerome A. Hogsette: Writing – original draft, Writing – review & editing, Visualization. Daniel L. Kline: Investigation, Resources, Validation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data from the study are available in the Supplementary file S1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2023.100113.

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