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New manuscripts of articles, operational or scientific notes, annual meeting abstracts, and page proofs should be sent to the Editor by e-mail attachment at xueamcd@gmail.com. The Editor will assemble the manuscripts for the assistant editor/subject editor or guest editor to conduct the peer review process.

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2023-2024 THE FMCA PRESIDENTIAL ADDRESS

James Richard Weaver

Anastasia Mosquito Control District, St. Augustine, FL 32092

I am so happy to be asked by the editor of the Journal of the Florida Mosquito Control Association to address the membership and provide a summary of the activities and accomplishments during my term as President of the association.

It has been an honor to be following in the footsteps of such giants in the field of mosquito control, who have held the office of President. As a Board member and then as President, I have worked with a great team of talented people including past President Sandra Fisher-Granger. Without the support of a strong Board and a talented and strong Director, this year would not have been as productive as it has been.

During my time as the President of the FMCA, we continued to do great things for our organization, Florida, and to a lesser extent the world. Public health is so very important and contributing to, and promoting this noble mission is imperative. Some of the important work accomplished by the Board, Director and membership are as follows.

The association was again expertly managed by CMC Associates with Karen Crawford serving as Executive Director. Under her leadership membership continues to grow, the finances are well managed, and the day-to-day operations go smoothly. The association, through Karen's hard work, was able to get some large districts to rejoin the association, having these important players involved in the FMCA is a must. The excellent event planning by Karen and her staff allowed the FMCA to hold world-class events.

Because of the fallout from the Florida legislature's order for the Office of Program Policy Analysis and Government Accountability (OPPAGA) study, the study findings and subsequent Bills offered in the Florida House and Senate that would limit powers of certain Special Districts, including Mosquito Control Special Districts our Legislative Committee, led by Keria Lucas, and the FMCA Board of Directors had their hands full trying to mitigate possible damages posed from these bills. The FMCA legislative committee, FMCA lobbyist, FMCA Public Relations firm, Special Districts, and FMCA members successfully advocated for the removal of adverse bill language to include the need to reauthorize special districts and for the FCCMC to develop model goals and performance measures as opposed to the districts themselves. The FMCA also helped get an increase of one million dollars in mosquito control State aid for the fiscal year budget 2024/2025.

The annual aerial meeting and fly-in was a great success and was held at Manatee County Mosquito Control District's new facility and as per usual Chris Lesser and Mark Latham did a great job hosting this event. Flying aircraft for mosquito control districts is not for the faint hearted, this event held by the FMCA annually allows pilots, aircraft mechanics, technicians, staff, and Directors to learn about changes to laws and regulations, safety, and sharing of new and changing technology. Next year (2025) the FMCA will be returning to Lee County Mosquito Control District for this event!

The DODD shorts courses continue to be a busy, educational, and fun event and are so popular they are bursting at the seams. This year's event featured Mosquito Wars, evening games geared for fun, fellowship, and some learning. Thank you to Committee Chair Shelley Whitehead and the DODD committee members.

Tallahassee days was more important this year than normal, due to the bills that were introduced (and subsequently passed) limiting the powers of mosquito control special districts. The event started with a two-hour program aimed at preparing participants for visiting their representatives' offices. At the Capitol we were able to once again co-host a news conference, arranged by Alia Strategic Group, this year highlighting the dangers of mosquito borne diseases. FMCA representatives from many districts met with dozens of House and Senate members and their staff. FMCA members also met with Department of Agriculture and Consumer Service Commissioner Wilton Simpson.

Wing Beats magazine is doing well under editor Dennis Moore, making a small profit but a big impact in the mosquito control world. This publication includes 3,500 printed copies per issue and a digital version published on the FMCA website. New in 2025 Buzz Words will be switching to a digital publication with Whitney Qualls's at the helm, this change should make the content more up-to-date and hopefully increase readership.

The annual meeting was held in Orland this year and was a great success. The Young Professional group

continues to grow and spends the Monday before the meeting, training and in fellowship. This group is so important, and the young professionals represented are the future of mosquito control and the FMCA. The poster competition was held again this year and was very well received by the membership. The presentations were very strong this year and are an important part of the meeting. New regional representatives and a Vice President were elected and the after-party, Havana Nights, was a blast. Also a hearty thanks to all the sponsors who help make this event possible.

In closing, I would like to give a well-earned thank you to the dedicated members of our Executive Board, including our President-Elect Jorge Ray, Vice President Peter Jiang, Past President Sandra Fisher-Granger, and Director Karen Crawford. Your hard work, commitment, and just plain being helpful have been instrumental in moving our association forward. I'd also like to thank our regional representatives, Member at Large, and Commissioner's representative for their hard work and dedication to the association. Included in these well-earned thank you 's are our industry members who generously provided stipends for first-time attendees of our Tallahassee Days event, as well as all the other events where they have lent their support to our association. This support has allowed us to engage and educate both current and new members about the critical role of mosquito control in our state.

As I pass the invisible gavel to our incoming president, Jorge Ray, I am sure he will be a great leader and keep the vision for the association alive and moving forward. I encourage all members to continue supporting the FMCA and working together to advance our mission of protecting the health and well-being of both Florida residents and our guests in the state.

PERSPECTIVES ON THE APPLICATION OF ENVIRONMENTAL DNA/RNA APPROACHES TO MOSQUITO SURVEILLANCE AND CONTROL IN FLORIDA

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ABSTRACT

Mosquito-borne diseases threaten four billion people globally, impacting public health and economies. Health, tourism, and agriculture in the United States are all affected by mosquitoes and the diseases they carry. Florida, in particular, faces increasing risks due to urban expansion, longer breeding seasons, and invasive mosquito species. The resurgence of locally contracted malaria in 2023, alongside threats like dengue, Zika, and chikungunya, highlights the urgent need for advanced monitoring. Beyond human health, mosquitoes also endanger Florida's livestock, pets, and wildlife, adding to economic and ecological concerns.

Environmental DNA/RNA (eDNA/eRNA), which captures genetic material from environmental samples, presents a groundbreaking solution. Already proven effective for tracking arthropod pests and disease vectors, eDNA can enhance mosquito surveillance by detecting species presence and assessing control effectiveness through DNA shed in the environment. Given Florida's advanced mosquito control network, the state offers an ideal testing ground for implementing eDNA approaches.

Integrating eDNA analysis into mosquito control strategies could revolutionize surveillance, providing a more efficient and cost-effective method for early detection. Additionally, eDNA/eRNA analysis of wastewater could enable real-time pathogen monitoring, while genomic tools could track insecticide resistance. eDNA and eRNA can be used together to obtain a comprehensive picture of biological communities. While eDNA captures both recent past and present evidence of an organism's presence, eRNA primarily reflects current biological activity. This complementary approach allows researchers to detect species and assess their biological functions. This paper reviews the latest advancements in eDNA technologies and explores their potential to improve Florida's mosquito surveillance, offering a powerful tool to enhance disease prevention, reduce economic losses, and strengthen public health efforts.

Key words: environmental samples, surveillance, mosquito control, pathogen monitoring, mosquito identification, mosquito quantification, eDNA, eRNA

INTRODUCTION

Globally, four billion people are at risk from mosquito-borne diseases. Mosquitoes are detrimental to many aspects of Florida's economy and the quality of life of citizens and visitors alike, placing burdens on tourism, health and economic activity (Aryaprema et al., 2023; Coatsworth et al., 2022; Kondapaneni et al., 2021; Moise et al., 2021). Continued urban

and agricultural development, warming temperatures and increased anthropogenic habitat use are changing the distributions of native mosquito species and the diseases they carry. Furthermore, invasive mosquito species are bringing new diseases to the US, with Florida being a particular hotspot. These include mosquito-borne dengue (DENV), Zika (ZIKV) and Chikungunya (CHIKV) viruses, and, in 2023, the first locally-contracted malaria outbreak in Florida in over two decades. Mosquito- and vector-borne diseases also affect Florida's livestock (e.g., horse and poultry industries), pets (e.g., dog heartworm disease) and wildlife.

Florida's warm, humid climate, consistent precipitation, and proximity to tropical regions allow mosquitoes to thrive (Bikangui et al., 2023; Campbell et al., 2021). Mosquitoes place a significant burden on human populations (Aryaprema et al., 2023; Coatsworth et al., 2022; Kondapaneni et al., 2021; Moise et al., 2021) with more than 900 mosquito-borne infections reported annually in Florida, over 2,000 cases across the US, and approximately 249 million cases worldwide each year. It is critical to establish and maintain robust surveillance systems (FDOH, Jan. 2025; WHO, Jan. 2025). Mosquitoes in Florida concentrate mainly in central and coastal areas of the state (Abílio et al., 2018; Wilke et al., 2021). Urban development in these economically active regions creates prime mosquito hotspots by introducing poor-quality standing water and reservoirs that support multiple mosquito species (Bikangui et al., 2023; Wilke et al., 2021). Reduced competition and fewer predators further boost mosquito populations(Moise et al., 2021). Key factors influencing mosquito densities include the number of household occupants, the presence of vegetation, and water management practices (Bikangui et al., 2023; Talbot et al., 2021; Wilke et al., 2021). Neighborhoods with lower socio-economic status (SES) tend to have higher mosquito densities due to inadequate waste disposal and fewer mosquito prevention measures (Talbot et al., 2021). However, factors such as decorative vegetation also create breeding sites, resulting in a risk of mosquito-borne diseases, including arboviruses, at all socio-economic levels (FDOH, Jan. 2025; Talbot et al., 2021; VDCI, Jan. 2025; WHO, Jan. 2025).

Environmental DNA (eDNA) is genetic material shed by organisms into their surroundings, such as water, soil, or air (Mauvisseau et al., 2022). eDNA is DNA recovered from environmental samples and has demonstrated great promise for accurately surveilling a broad range of species from microbes to mammals, including many arthropod pest and disease vector species. DNA and RNA leave multi-cellular organisms and enter the environment through various mechanisms including shedding (skin, hair, scales, etc.), molting (insect exoskeletons, snake skins, etc.), defecation, bodily fluids (blood, mucus, urine etc.), respiration, gamete release, and decomposition of deceased individuals (Mauvisseau et al., 2022; McCauley et al., 2024). This DNA or RNA can be used to monitor the occurrence of specific species, including mosquitoes.

eDNA monitoring has been applied to wildlife, invasive species, agricultural pest species, fisheries and pathogens in habitats across the globe (Farrell et al., 2021a; Miaud et al., 2019; Nousias et al., 2025; Thomsen and Willerslev, 2015). Non-insect DNA has even been retrieved from mosquitoes and other biting insects, and used to detect the presence of humans and other terrestrial vertebrates in natural and urban environments (Hopken et al., 2021; Massey et al., 2022; Trájer, 2018). eDNA/eRNA approaches outperform conventional detection methods, and can give advanced warning of disease outbreaks (Farrell et al., 2021a; Miaud et al., 2019; Nousias et al., 2025; Thomsen and Willerslev, 2015). Refinement of eDNA approaches for mosquito monitoring and their widespread implementation into existing mosquito control strategies will complement and enhance current mosquito monitoring efforts within Florida (Fig. 1). Given Florida's technologically advanced and widespread mosquito surveillance and control network, the state offers an ideal testing and implementation ground for mosquito eDNA approaches. eDNA can be used to innovatively monitor the occurrence of mosquito species and the effectiveness of control measures. For example, a fixed air eDNA sampling network could generate continuous monitoring and quantification of mosquito species throughout the state, while aquatic eDNA sampling could be used to identify breeding hotspots in water bodies ranging in size from saltmarshes to storm drains and containers. Furthermore, when applied to human wastewater (sewage), eDNA/eRNA approaches can likely be utilized to monitor mosquito-borne pathogens (Farrell et al., 2021a). Genomic eDNA applications also hold promise for monitoring the spread of insecticide resistance among mosquito populations from environmental sampling (Nousias et al., 2025).

This paper reviews the application of eDNA technologies in mosquito surveillance, highlights recent advancements in eDNA/eRNA techniques, and explores how these technologies can be integrated into the routine surveillance efforts of mosquito control districts to monitor Florida's mosquito species and pathogens of concern. Such novel rapid surveillance approaches will enable more cost-effective targeted mosquito control and disease mitigation measures and advance our ability to manage and reduce mosquito burdens, including

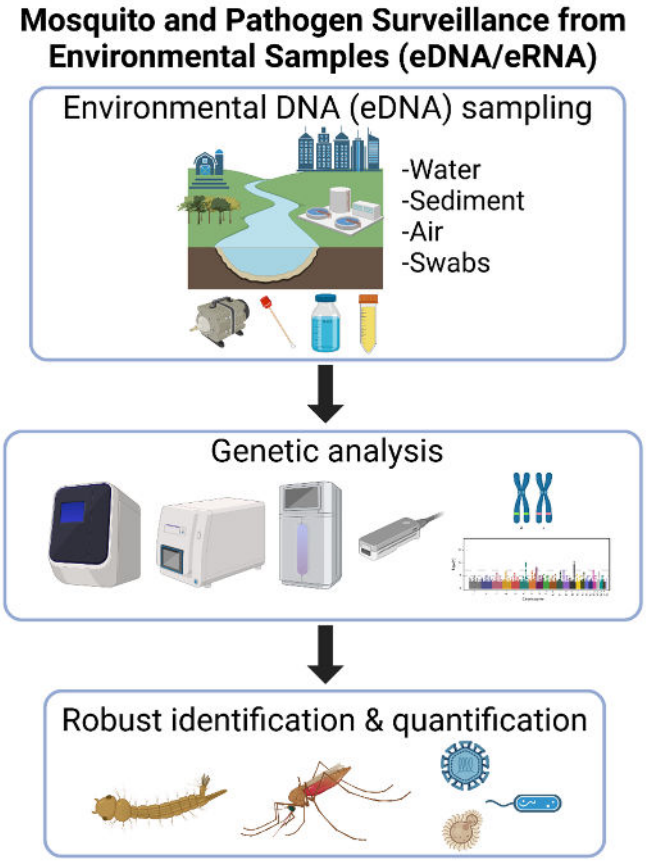

Figure 1. Schematic overview of mosquito (water, sediment, swab or air) and mosquito-borne pathogen (wastewater) detection, quantification, and genetic analysis from environmental samples. Once optimized, eDNA/eRNA approaches can provide robust temporal and spatial information to inform mosquito control and disease mitigation efforts. Schematic was created in BioRender for this paper. Duffy, D. (2025), https://BioRender.com/o8c0ho4.

economic impacts and mosquito-borne diseases (Farrell et al., 2021a; Nousias et al., 2025).

Individual species to whole-biome-level eDNA detection technologies

eDNA sample collection is straightforward and easily taught, with many eDNA studies successfully utilizing citizen scientists or school children as sample collectors. eDNA extraction methodologies vary by sample type, but all require the purification of DNA from the environmental substrate. Generally, filtration is the most commonly applied approach. Aquatic samples (milliliters to liters) are filtered (e.g., using 0.22µM filters) and DNA is then extracted from the filter. Sediment samples can be suspended in aqueous solution and DNA extracted and recovered by filtration or centrifugation. Airborne DNA can readily be recovered by filtration using a fan or vacuum pump to pass air through a filter. Samples can be collected and filtered on site, and DNA/RNA stabilization solution added for transport back to the lab.

Once extracted from the environmental substrate, eDNA/eRNA can be investigated with any conventional DNA/RNA analytical approach. Commonly, tools like quantitative PCR (qPCR), digital PCR (dPCR) or reverse-transcription (RT) qPCR/dPCR are utilized for species-specific or pathogen-specific applications. qPCR/dPCR approaches are best suited to investigating small numbers of species. For broader animal species assessments, metabarcoding is commonly used. Small informative stretches of DNA (barcodes) that can be used to differentiate between different genera or species are first amplified with conventional PCR, and the resulting PCR products (barcodes) are then sequenced using a next-generation sequencing platform. Metabarcoding has also been widely applied to microbial environmental studies (Abdelfattah et al., 2018). However, microbial metagenomic studies are becoming more widespread (Ko et al., 2022; Simmonds et al., 2024; Venter et al., 2004). In metagenomics, all eDNA/eRNA present in a sample is whole-genome sequenced without the need for specific barcodes. Metagenomics, also referred to as shotgun sequencing, is now gaining traction as an applicable approach to multi-cellular species eDNA studies (plant, animal and fungal). Indeed, metagenomics can readily simultaneously recover DNA/RNA from viruses to vertebrates (Nousias et al., 2024; Nousias et al., 2025), enabling the investigation of pathogens, vectors and hosts within the same environmental sample.

PCR-free enrichment approaches are also possible. For example, panels of hybridization probes can be designed to bind to specific target DNA/RNA sequences of interest, with only the target regions being sequenced. While originally designed for more traditional samples (e.g. tissue, blood, whole organism), this approach has been successfully applied to air, water and sediment eDNA (Nousias et al., 2025; Whitmore et al., 2023). The development of a hybridization probe-based enrichment panel for the detection and routine surveillance of all mosquito species that occur in Florida is highly feasible.

The most appropriate approach depends on study specifics and cost implications. For routine mosquito surveillance, barcodes or hybridization probe capture panels would likely prove to be the most feasible and cost-effective approach to simultaneously monitor all species/genera known to be present in Florida. For continual monitoring of a small number of mosquito species of concern (e.g. 1-10 species), qPCR/dPCR would be the

most rapid approach. While not covered in detail here, it should be noted that on-the-spot field-based eDNA testing for species is being developed using a number of detection technologies, and these technologies could also be readily converted for mosquito-surveillance applications.

Sampling and genetic material recovery have been optimized from a broad range of Floridian ecosystems: aquatic samples from ocean, freshwater and saltmarsh, air samples from coastal and inland field locations and indoor settings, sediment sampling (beach sand, soil and aquatic sediments), and surface swabs from any field surface (Farrell et al., 2022; Farrell et al., 2021b; Koda et al., 2023; McCauley et al., 2024; Nousias et al., 2025; Whitmore et al., 2023). As part of our biodiversity assessments, mosquito eDNA was recovered by these approaches (Farrell et al., 2022; Farrell et al., 2021b; Koda et al., 2023; McCauley et al., 2024; Nousias et al., 2025; Whitmore et al., 2023). These validated approaches can be deployed to recover genetic material from all Florida habitats, and could be readily applied to targeted eDNA-based mosquito surveillance activities.

The promise of eDNA for complementary pan-mosquito species monitoring

eDNA approaches greatly complement and often surpass existing monitoring approaches across a wide range of fields. Mosquito surveillance in Florida, and globally, stands to benefit from the application of eDNA technologies. By recovering the trace amounts of DNA shed by organisms into their environment, eDNA approaches have been shown to enable cost-effective, scalable species-specific identification with robust temporal and spatial resolution. eDNA/eRNA-based species and pathogen monitoring generally outperforms conventional monitoring approaches, and have been applied in diverse sectors from fisheries to endangered and invasive species, and to human and wildlife health (Farrell et al., 2021a; Farrell et al., 2022; Nousias et al., 2025; Thomsen and Willerslev, 2015). Given the broad range of species found within Florida, and the variability of species affinities for existing mosquito monitoring traps, eDNA approaches hold great promise for more species agnostic (i.e., less biased) simultaneous monitoring of all native and invasive mosquito species currently found in Florida. Traditional mosquito surveillance methods, while reliable, are labor-intensive, require expertise, and depend on direct specimen collection (Sakata et al., 2022). These methods are challenging in terms of efficiency in large or hard-to-access areas and are facing emerging limitations due to insecticide resistance in adult mosquitoes (Giordano et al., 2020; Sakata et al., 2022). eDNA has become a promising complementary or alternative approach, offering significant advantages. eDNA research has effectively addressed many entomology questions (Allen et al., 2021; Schneider et al., 2016; Uchida et al., 2020; Valentin et al., 2020; Valentin et al., 2018) and offers significant advantages for monitoring mosquito populations and related diseases. It enables the detection of mosquitoes at various developmental stages, even in the absence of live specimens, improving the ability to identify breeding sites in hard-to-access areas (Sakata et al., 2022). eDNA sampling is more efficient and non-invasive than traditional methods, reducing the need for extensive expertise or direct specimen collection (Sakata et al., 2022; Schneider et al., 2016). Additionally, technologies such as automated samplers and light aircraft- and drone-based sampling extends the reach of eDNA while reducing sampling burdens (Nousias et al., 2025). Furthermore, eDNA can provide a more accurate and comprehensive picture of mosquito presence, including rare or elusive species, while overcoming challenges like insecticide resistance (Sakata et al., 2022; Sengupta et al., 2022; Wittwer et al., 2024). This makes eDNA a valuable tool for both monitoring and controlling mosquito-borne threats.

Aquatic and sediment mosquito eDNA studies to date

Despite the enormous potential for eDNA approaches to contribute to routine mosquito surveillance and control activities, few mosquito surveillance-focused eDNA studies have been carried out to date (Boerlijst et al., 2019; Gutiérrez-López et al., 2023; Kristan et al., 2023; Krol et al., 2024; Krol et al., 2019; Nousias et al., 2025; Odero et al., 2018; Sakata et al., 2022; Schneider et al., 2016) (Table 1). Mosquito eDNA studies have almost exclusively focused on using aquatic sampling (Table 1), though Boerlijst et al. (2019) also examined sediment sampling. In artificial breeding conditions, mosquito eDNA could be detected by as few as one-second stage instar larva in 1 L of water (Kristan et al., 2023).

eDNA research has revealed its utility across mosquito developmental stages and habitats and has broad global applicability (Table 1). In Japan, mosquito eDNA was detected at all stages, with peaks after egg hatching and pupa emergence (Sakata et al., 2022). In Europe, mosquito eDNA enabled the detection of invasive species despite high degradation rates, persisting for up to 25 days and outperforming traditional surveys (Schneider et al., 2016). In African plantations and rainforests, eDNA of multiple mosquito genera, including first-time detections, was identified, even in disturbed environments (Gutiérrez-López et al., 2023). The global

Table 1. Studies that utilized eDNA technologies for mosquito and mosquito-borne virus detection.

Study	Location	Approach	Link
Mosquitoes:			
Nousias et al., **2025**	Florida	Air eDNA, metagenomics	www.researchsquare.com/article/rs-5953812/v1 (pre-print)
Wittwer et al., **2024**	Germany	Water eDNA, qPCR & microfluidic array technology	https://pubmed.ncbi.nlm.nih.gov/39364359/
Krol et al., **2024**	The Netherlands	Water eDNA, ddPCR	https://pmc.ncbi.nlm.nih.gov/articles/PMC10826093/
Gutiérrez-López et al., **2023**	Gulf of Guinea, Africa	Water eDNA, metabarcoding	https://pubmed.ncbi.nlm.nih.gov/37183666/
Kristan et al., **2023**	Laboratory	Water eDNA, qPCR	https://pubmed.ncbi.nlm.nih.gov/36792622/
Sakata et al., **2022**	Laboratory	Water eDNA, qPCR	https://pubmed.ncbi.nlm.nih.gov/35947597/
Sengupta et al., **2022**	Global	Review	https://pubmed.ncbi.nlm.nih.gov/36419798/
Krol et al., **2019**	South Africa	Water eDNA, qPCR & metabarcoding	www.frontiersin.org/journals/ecology-and-evolution/articles/10.3389/fevo.2019.00260/full
Boerlijst et al., **2019**	Caribbean	Water and sediment eDNA, metabarcoding	www.frontiersin.org/journals/ecology-and-evolution/articles/10.3389/fevo.2019.00240/full
Odero et al., **2018**	Laboratory	Water eDNA, qPCR	https://pubmed.ncbi.nlm.nih.gov/29911186/
Schneider et al., **2016**	Europe	Water eDNA, qPCR	https://pubmed.ncbi.nlm.nih.gov/27626642/
Mosquito-borne viruses:			
Birnberg et al., **2020**	Spain	Arboviruses in mosquito saliva, FTAs & RT-PCRs	https://pubmed.ncbi.nlm.nih.gov/32121402/

success of eDNA applications demonstrates the potential for improving mosquito surveillance and vector-borne disease monitoring. Minimal water volumes are required for detection, and its application to malaria vectors highlights the potential of eDNA for disease monitoring (Mapua et al., 2024; Wittwer et al., 2024). However, limited eDNA research exists in regions like Florida with high numbers of mosquito vectors, underscoring the need for further field studies to explore environmental factors such as water chemistry and turbidity, in order to address local challenges and improve detection capabilities.

Airborne DNA for adult mosquito surveillance

Airborne DNA is emerging as a frontier in eDNA research. Air, like water and sediment, is rich in eDNA, which therefore opens the possibility of direct air sampling for mosquito surveillance (Lynggaard et al., 2024; Nousias et al., 2025). UV radiation degrades eDNA, which may limit spatial and temporal detection in tropical and subtropical regions like Florida. However, mosquito airborne eDNA was recoverable from Florida field sites (Nousias et al., 2025). eDNA approaches can quantify the level of mosquito DNA present in each sample, enabling comparison between sites (spatial variation) and across time (temporal variation). Within Florida, fine scale (sub-4km) differences were observed in airborne mosquito eDNA loads between a coastal forest hammock and a more exposed coastal beach site (Nousias et al., 2025). Differences in mosquito loads were also observable at the same Florida sites over time and between different mosquito genera (Nousias et al., 2025). Mosquito DNA was recoverable from the beach and forest sites (Nousias et al., 2025) despite them being in the Anastasia and East Flagler Mosquito District control zones, respectively.

Both long-read and short-read metagenomics successfully detected mosquito eDNA from air samples (Nousias et al., 2025). Furthermore, long-read barcodes for mosquito identification from traditional (whole animal) samples have already been validated (Hartke et al., 2022). eDNA could be recovered by a variety of sampling approaches, including direct filtration of eDNA from the air (indoor and field air), or swabbing of surfaces in the environment to recover settled airborne DNA. Controlled studies are essential to determine deposition, degradation, and dispersal rates. These approaches detected eDNA from diverse metazoan species, particularly arthropods, which were the most abundant animal phylum across aquatic, air, and sediment samples (Nousias et al., 2024; Nousias et al., 2025). Airborne DNA holds great promise for mosquito surveillance, but further research is needed to understand the relationship between airborne DNA quantity and local mosquito biomass.

eDNA mosquito surveillance applications

US federal agencies are beginning to consider eDNA approaches for mosquito surveillance and control, such as the use of water eDNA sampling by the USGS to survey for the invasive southern house mosquito (*Culex quinquefasciatus*) in Hawai'i (USGS, Jan. 2025). However, eDNA tools for mosquito surveillance are currently massively underutilized, not just at the federal level, but also the state level, including within Florida. The range of substrates which can be sampled for eDNA (e.g. aquatic, sediment and air), and the breadth of downstream technologies which can focus from individual species to whole biomes (e.g. species-specific qPCR to metagenomics) means that there are diverse opportunities for the integration of eDNA approaches to mosquito surveillance. The most appropriate eDNA sampling and analysis technologies will depend on specific surveillance goals. These could range from surveillance of small-scale standing water, e.g., assessing individual storm drains or vegetation pools, to habitat-level assessments, e.g., determining the specific sub-areas of a saltmarsh responsible for the largest volume of *Aedes taeniorhynchus* production. Similarly, air eDNA approaches could be applied to indoor locations to assess hyper-localized mosquito presence, or air eDNA monitoring networks could be established at landscape scales in rural or urban locations to identify those areas most in need of enhanced mitigation and control measures (Nousias et al., 2025). eDNA approaches could also be used to understand population dynamics and the duration between application of control measures and the post-treatment recovery of mosquito populations. As well as assessing specific mosquito species and their eDNA abundances, tools to monitor insecticide resistance markers from mosquito samples could be readily adapted for use with eDNA samples to enable broader-scale surveillance.

eDNA approaches can complement more traditional mosquito surveillance. In many cases it can be advantageous to couple eDNA monitoring and conventional monitoring approaches, with each providing complementary data. For example, larval dipping can provide accurate estimates in small areas with high mosquito numbers, while eDNA can detect mosquito presence in large areas with low larval numbers (Barnes and Turner, 2016; Krol et al., 2024; Nousias et al., 2025). Legacy mosquito presence can also be detected via eDNA still present in the water or sediment after adult emergence (Boerlijst et al., 2019; Dejean et al., 2011; Krol et al., 2024). It is known that eDNA persists in the environment for varying durations depending on abiotic and biotic factors, including microbial activity, temperature, UV exposure, and pH levels. Generally, residual eDNA can persist for one to four days until it is fully degraded (Barnes et al., 2014; Krol et al., 2024; McCauley et al., 2024; Strickler et al., 2015; Zhao et al., 2023). Differences in eDNA degradation rates in aquatic ecosystems should also be considered for eDNA mosquito surveys. For example, mosquito eDNA could be expected to persist for shorter durations in saltmarshes compared with freshwater habitats. If tightly restricted temporal and spatial information is a requirement for a particular survey then eRNA can be utilized instead of eDNA, as single-stranded RNA is a less stable molecule that degrades more rapidly than double-stranded DNA (Farrell et al., 2021a; Giroux et al., 2022). Conversely, eDNA can persist in the environment for prolonged periods (up to 2 million years (Kjær et al., 2022)) if conditions promote DNA stability, such as certain lakebed and deep-sea sediments, ice and frozen sediments. Thus, ancient eDNA (aeDNA) may provide novel opportunities for studying ancient mosquito genetics, distributions and abundances.

Wastewater surveillance for arboviruses

The application of eDNA/eRNA approaches to wastewater systems to monitor arbovirus loads is also currently underutilized. Since 2019, aquatic and wastewater monitoring programs have been successfully initiated in many countries to monitor wildlife and human infectious pathogens (Ahmed et al., 2020; de Jonge et al., 2022; Farrell et al., 2021a; Kumar et al., 2020; Miaud et al., 2019; Mtetwa et al., 2022; Nousias et al., 2024; Randazzo et al., 2020). While pathogens monitored to date tend to be directly transmissible, if a sufficient proportion of a population is infected with an arbovirus

or other mosquito-borne pathogen it is highly likely that these infections could similarly be monitored through wastewater eDNA/eRNA sampling. Wastewater eDNA/eRNA-based monitoring has been shown to give advanced warning of disease outbreaks, with directly transmitted pathogens being detected weeks in advance of increased hospitalizations (Ahmed et al., 2020; de Jonge et al., 2022; Farrell et al., 2021a; Kumar et al., 2020; Miaud et al., 2019; Mtetwa et al., 2022; Nousias et al., 2024; Randazzo et al., 2020). The fact that many arboviruses are RNA viruses is not a limitation to wastewater eDNA surveillance. Rather, wastewater monitoring for RNA viruses has been well established, with largescale effort in optimization of such approaches during the SARS-CoV-2 pandemic. RNA and DNA virus surveillance has also been successfully conducted from indoor and outdoor air eDNA/eRNA sampling, and aquatic field sampling (Lednicky et al., 2020; Miaud et al., 2019; Nousias et al., 2024; Nousias et al., 2025; Whitmore et al., 2023). In addition to viral load detections, wastewater eDNA/eRNA monitoring has been shown to be a reliable epidemiological tool for tracking viral variants and temporal shifts in the predominant variant afflicting a population (genomic surveillance). Like for mosquito species themselves, the development of hybridization probe-based enrichment panels for the detection of all arboviruses of concern that occur in Florida for routine wastewater-based surveillance is highly feasible.

Specific research into the number of arboviruses recoverable from the main sources of human material in wastewater (e.g. urine, feces, blood, GI tract cells) are required, as are studies on the number of infected individuals required for robust wastewater detection. However, wastewater monitoring has proven effective even for pathogens with airborne transmission, suggesting that infected individuals shed viral particles into wastewater systems, even if bodily secretions are not the primary mode of transmission. Taken together, eDNA approaches are likely to enhance our ability to monitor both mosquitoes and the pathogens they transmit.

Florida's native and invasive mosquito species and arboviruses

Mosquitoes are primary vectors for pathogen transmission through interactions between humans and other species. Of particular burden to coastal communities are saltmarsh species like *Ae.* t*aeniorhynchus* (Dale and Xue, 2024), while Florida's continued urbanization has been shown to favor the proliferation of storm drain breeding species such as *C. quinquefasciatus* (Wilke et al., 2021). Species such as *C. quinquefasciatus* and *Aedes albopictus* are relatively abundant in Miami-Dade County (Wilke et al., 2022). Originating from maritime ports for overseas travel and trading, they are responsible for spreading various diseases, including DENV, ZIKV, yellow fever (YFV), and CHIKV viruses (Wilke et al., 2022). CHIKV, ZIKV, and DENV are transmitted primarily by *Aedes aegypti* and *Ae. albopictus* (FDOH, Jan. 2025). DENV serotypes (DENV-1 to DENV-4) that cause dengue fever are closely related to other flaviviruses, including WNV and YFV and are also spread by *Ae. aegypti* (FDOH, Jan. 2025; VDCI, Jan. 2025). *Ae. albopictus* has displaced *Ae. aegypti* in northern parts of Florida as detected by Parker et al. (2019); however, *Ae. aegypti* has started reappearing in non-coastal regions of peninsular Florida (Parker et al., 2019). *Ae. albopictus* is well-adapted to human habitats and thrives in urban and semi-urban areas. Its breeding sites are often linked to artificial containers and poor waste management, which serve as breeding grounds for the species and as hotspots for transmitting diseases such as ZIKV (Bikangui et al., 2023; McAllister et al., 2020; Talbot et al., 2021; Wilke et al., 2021; Wilke et al., 2022). During the 2016 Zika epidemic in Miami, these reservoirs played a significant role in the rapid spread of the virus (McAllister et al., 2020). However, Florida's swift response, characterized by intensive control activities and strategic interventions, was instrumental in quickly curbing the epidemic (McAllister et al., 2020). Although *Ae. albopictus* was rarely observed by Wilke et al. (Wilke et al., 2022), its presence in other regions signals its expanding range (Talbot et al., 2021; Wilke et al., 2022). Both species display high vector competence for transmitting arboviruses such as ZIKV, DENV, and CHIKV (Talbot et al., 2021; Wilke et al., 2022). The affinity of *Ae. aegypti* for indoor and peri-domestic environments underscores its close association with human populations in urban areas experiencing high precipitation (Talbot et al., 2021; Wilke et al., 2022; Yang et al., 2021). Heavy rainfall facilitates the creation of breeding sites in artificial containers, supporting its proliferation (Yang et al., 2021). In contrast, *Ae. albopictus* tends to thrive in more vegetative, semi-natural habitats across suburban and rural areas (Talbot et al., 2021; Wilke et al., 2022; Yang et al., 2021). Despite the density of *Ae. albopictus* surpassing that of *Ae. aegypti*, both coexist within overlapping urban niches (Yang et al., 2021), and exhibit heightened activity in Florida during the rainy and warm season, spanning from May to October (Yang et al., 2021).

Another notable species, *Aedes tortilis*, was first recorded in the United States in Key West in 1945 and has since spread along southern Florida's coastal regions, particularly the Atlantic Coast (Heinig et al., 2023). By 2021, researchers identified this species in Collier County,

inhabiting mangrove habitats and residential areas (Heinig et al., 2023). *Culex tarsalis* has a limited presence in Florida, but serves as a primary vector for Western equine encephalomyelitis, St. Louis encephalitis, and West Nile virus (Heinig et al., 2023). Its detection in South Florida in 2021 has raised public health concerns regarding endemic West Nile virus (Heinig et al., 2023). This suggests that this species has yet to establish a permanent population, possibly due to unfavorable conditions like Florida's warm winter temperatures (Heinig et al., 2023). Another emerging concern in Florida is the Keystone virus (KEYV), transmitted by *Aedes atlanticus* (Elbadry et al., 2023). While underrecognized, this virus poses public health risks, including potential neuroinvasive diseases (Elbadry et al., 2023). Malaria, a common mosquito-borne illness both nationally and globally, is transmitted by *Anopheles* mosquito species (Elbadry et al.). *C. quinquefasciatus* can also cause Rift Valley fever and St. Louis encephalitis (FDOH, Jan. 2025). Eastern equine encephalitis, spread by *Culiseta melanura,* is also common in Florida (FDOH, Jan. 2025). The arboviruses shown in Table 2 are also thriving in ecosystems such as the Everglades in Southern Florida (Table 2).

Summary - Integration of eDNA/RNA techniques in Florida

Effective mosquito control is essential to the state of Florida for the health and quality of life of its residents and visitors and for its continued economic and agricultural success. Mosquito control is a key component underpinning Florida's population growth, which fueled construction sector growth and employment, with more residents and workers being attracted to the state since the introduction of control measures. Furthermore, over 105 million tourists visit Florida each year (Florida Governor's Office news release, Nov 2023), with 1 in 6 jobs in the state being dependent on this sector (UF IFAS and Center for Public Health Education, 2016). In 2020 alone, tourism contributed $101.9 billion to Florida's Gross State Product (The 2021 Economic & Fiscal Impact of Tourism in Florida, Rockport Analytics). Prior to mosquito control efforts initiated in the 1950s, mosquitoes posed a serious threat to the livelihood of Floridians and a serious barrier to growing the state's tourism sector. They can deter tourism, disrupt outdoor activities, and negatively impact agriculture, leading to significant economic losses. However, continued urbanization, climate change, and the emergence of invasive mosquito species demand ongoing innovation in surveillance and control strategies. The control of mosquitoes has played a crucial role in attracting more residents, increasing job numbers, increasing State revenue and improving the health and quality of life of residents and visitors. Therefore, maintaining and advancing mosquito control and research efforts is essential to reducing the burden of mosquitoes in Florida and addressing emerging threats. Novel invasive species, the diseases they carry, and the shifting distribution of mosquito populations due to increased urbanization pose ongoing challenges. Integrating eDNA technologies into Florida's established mosquito surveillance and control programs will enhance their resilience, enabling earlier detection and more effective management of mosquito populations. Such eDNA integration, pioneered in Florida, would then provide an integration roadmap for other states and countries. By providing accurate, timely data on species presence and distribution, these technologies can support the Florida Department of Health (FDOH) and the Florida Department of Agriculture and Consumer Services (FDACS) in monitoring public health and economic risks and implementing control measures efficiently. Routine implementation of eDNA technologies will provide pre-emptive warning of population increases and evasion of control measures, and will facilitate tailored targeted responses as the state's mosquito profile continues to change in the face of longer breeding seasons and increased urbanization. These methods offer a non-invasive, scalable, and potentially cost-effective means of monitoring mosquito populations and the pathogens they carry.

However, significant challenges must be addressed before widespread adoption of mosquito eDNA monitoring. Sample collection and transportation can be logistically complex, requiring standardized protocols to minimize DNA degradation. We recommend the use of on-filter DNA stabilization solutions. Cross-contamination risks must be carefully managed to prevent false positives, while limits of detection must be clearly defined to ensure reliable data interpretation. Additionally, distinguishing between residual DNA from dead mosquitoes and active populations will be a key hurdle in accurately assessing real-time mosquito presence. To maximize the value of eDNA/eRNA integration, further research should focus on refining sampling methodologies, improving sensitivity and specificity, and developing best practices for field implementation. Additionally, studies exploring the application of eRNA for detecting active arbovirus infections in mosquito and human populations could enhance disease surveillance efforts. By optimizing eDNA approaches through localized trials and integrating them into Florida's mosquito control framework, the state can strengthen its ability to detect, track, and respond to mosquito threats more effectively. eDNA/eRNA-based

Table 2. Summary of current and historical arboviruses transmitted by mosquitoes in Florida, originating from local or travel-related infection.

Arbovirus (Abbreviation)	Taxonomy	Potential Host	Vector	Acquisition	References
Everglades virus (EVEV)	*Alphavirus*, Togaviridae	Hispid cotton rat, cotton mouse, dogs, human	*Culex cedecei*	Local	Bigler 1969, Lord et al. 1973, Coffey et al. 2006, Cadena et al. 2023.
Keystone virus (KEYV)	*Orthobunyavirus*, Peribunyaviridae	Hispid cotton rat, rabbit, squirrels, human	*Aedes atlanticus* *Culex cedecei*	Local	Jennings et al. 1970, Bigler et al. 1974, Fish et al. 2021, Henry et al. 2022.
Gumbo Limbo virus (GLV)	*Orthobunyavirus*, Peribunyaviridae	Hispid cotton rat	*Culex cedecei*	Local	Bigler et al. 1974, Fish et al. 2021.
Shark River virus (SRV)	*Orthobunyavirus*, Peribunyaviridae	Hispid cotton rat	*Culex* spp. *Culex cedecei*	Local	Fields et al. 1969, Fish et al. 2021.
Mahogany Hammock virus (MHV)	*Orthobunyavirus*, Peribunyaviridae	Hispid cotton rat	*Culex cedecei*	Local	Fish et al. 2021.
Tensaw virus (TENV)	*Orthobunyavirus*, Peribunyaviridae	Hispid cotton rat, rabbit, raccoon	*Anopheles crucians* *Culex cedecei*	Local	Mitchell et al. 1996, Anderson et al. 2022.
St. Louis encephalitis (SLE)	*Orthoflavivirus*, Flaviviridae	Birds, humans	*Culex pipiens* *Culex quinquefasciatus* *Culex tarsalis* *Culex nigripalpus*	Travel	Diaz et al. 2018, Ottendorfer et al. 2009.
Chikungunya virus (CHIKV)	*Alphavirus*, Togaviridae	Wild primates, humans	*Aedes aegypti* *Aedes albopictus*	Travel	Reiskind et al. 2008.
Dengue virus (DENV)	*Orthoflavivirus*, Flaviviridae	Bats, humans	*Aedes aegypti* *Aedes albopictus*	Travel	Rowe et al. 2023, Richards et al. 2012, Rey 2014, Gwee et al. 2021.
Eastern equine encephalitis virus (EEEV)	*Alphavirus*, Togaviridae	Passerine birds, horses, pigs, humans	*Culiseta melanura*, *Coquillettidia perturbans*, *Aedes cinereus*, *Aedes canadensis*	Travel	Banda and Samanta 2023, Mundis et al. 2022.
Malaria	*Plasmodium* Plasmodiidae	Primates (monkeys, gorillas), humans	*Plasmodium vivax* *Anopheles culicifacies*	Travel	Blackburn et al. 2023, Wilke et al. 2024.
Rift Valley fever virus (RVF)	*Phlebovirus*, Phenuiviridae, Bunyaviridae	Humans	*Culex quinquefasciatus*	Travel	Turell et al. 2013.
West Nile virus (WNV)	*Orthoflavivirus*, Flaviviridae	Passerine birds, mosquitoes, alligators, humans	*Culex nigripalpus* *Culex quinquefasciatus*	Travel	Day et al. 2015, Klenk et al. 2004.
Yellow fever virus (YFV)	*Orthoflavivirus*, Flaviviridae	Primates, humans	*Aedes aegypti*	Local	Damal et al. 2013.
Zika virus	*Orthoflavivirus*, Flaviviridae	Mice, cottontail rabbit, racoon, rodents, northern mockingbird, humans	*Aedes aegypti*	Travel	Philip et al. 2019, Stenn et al. 2019.

surveillance advancements will provide preemptive warnings of mosquito population shifts, improve targeted control measures, and enhance Florida's resilience against evolving mosquito-borne disease risks.

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A REVIEW OF LETHAL OVITRAPS FOR MANAGEMENT OF URBAN *AEDES* MOSQUITO VECTORS

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ABSTRACT

Due to rapid development and population growth, the container-inhabiting *Aedes* mosquitoes often lead to local public health problems. Moreover, the increasing detection of insecticide resistance in vector mosquitoes, along with growing environmental concerns, complicates control efforts. This paper is an overview of physical/mechanical and environmentally friendly lethal ovitraps for monitoring and controlling the container-inhabiting *Aedes* mosquitoes. Most ovitraps kill the larvae or adults and have been used as control tools for many years. These traps are mass ovitrapping, LOK autocidal ovitraps, Zeichner lethal ovitrap, sticky ovitrap, autocidal gravid ovitrap, gravid *Aedes* trap (GAT), autodissemination mosquito trap (In2Care), and Inzecto dual-action lethal ovitrap. Each trap has its advantages and disadvantages. The use of lethal ovitraps for surveillance and control of container-inhabiting mosquitoes is further addressed in this article.

Key words: Lethal ovitraps, immature control, container-inhabiting mosquitoes

With the global presence of Dengue, the recent emergence of Chikungunya and Zika, and the continued presence of yellow fever, control of urban *Aedes* container mosquitoes is imperative (CDC 2024). These disease organisms are primarily vectored in urban areas and require the control of the vector mosquitoes, primarily in the genus *Aedes*, i.e., *Aedes aegypti* Linn. and *Ae. albopictus* Skuse. These urban *Aedes* mosquitoes develop in small water-holding containers that are frequently found around residences, particularly where drinkable water is scarce and needs to be stored.

There is now concern that a container *Anopheles* mosquito will transmit Malaria in urban cities throughout Africa (Faulde et al. 2014). Even though Malaria is a very important disease vectored by *Anopheles* mosquitoes, Malaria vectors are not usually found developing in small containers. However, the primarily urban *Anopheles* mosquito, *Anopheles stephensi* Liston, does develop in small containers. This species is currently expanding its range from Asia into Africa (Faulde et al. 2014). It is estimated that an additional 126 million people will be at risk of getting Malaria due to the geographic expansion of this competent Malaria vector. Similar to the habitat of *Aedes* mosquitoes, *An. stephensi*, is typically found developing in small containers around urban residences. This is of serious concern in exposing urban and suburban residents to Malaria transmission, which currently is a predominantly rural disease in Africa (Faulde et al. 2014). Consequently, the concept of urban mosquito vectors needs to be expanded to include not only the urban *Aedes* but also the urban *Anopheles*.

The standard chemical control method for urban mosquitoes in most developed countries is peridomestic space spraying with short-lived insecticides to knock flying mosquitoes out of the air. These space sprays are best applied when the sun has set and when there is an inversion layer that keeps the spray close to the ground within the flight zone of the mosquito. Of course, for urban *Aedes* mosquitoes, their peak flight time is during the day rather than at night. Consequently, the time of space spraying does not coincide with the peak activity periods of urban *Aedes* vectors. In many parts of the world, residual adulticides are used on vegetation and in structures to kill resting mosquitoes. This method can effectively kill vectors of dengue and Malaria. The time of application is less important for residual insecticides than for space sprays. Both space sprays and residual treatments for mosquito control are labor intensive and expensive to utilize. Training of technicians to correctly apply these types of insecticides is important but is difficult to implement in order to provide effective management programs.

There is an important need for novel control measures that target urban *Aedes* mosquitoes and limit the

use of costly and sometimes environmentally unfriendly insecticide applications. These novel control measures should be reasonably priced for low-income, resource-poor areas of the world and need to be long-lasting and easily implemented.

Researchers have concentrated on the oviposition behavior of urban mosquitoes as a weak point in their life cycle that could be utilized for control using lethal ovitraps. Although the concept is much older, over the last thirty years or more, lethal ovitraps have been developed and modified as a simple way to use the oviposition behavior of mosquitoes as a method of control. Lethal ovitraps are small containers used by urban mosquito vectors as oviposition sites.

Emptying containers. The concept of lethal ovitraps began centuries ago, with people being advised to empty water containers where mosquitoes could develop. Most public health programs ask residents to empty containers in order to prevent larval mosquito development (Lloyd et al. 2018). When larvae developing in containers are killed by dumping out the water, that container becomes a lethal ovitrap. The concept is that mosquitoes lay their eggs in small containers around places where people live and work. If containers are emptied, any developing larvae would then be killed by simply dumping the water on the ground. This method is also known as the tip-and-pour method. Draining each container is critical because urban *Aedes* mosquitoes utilize skip oviposition to place their eggs (Davis *et al.* 2016). This results in the presence of mosquito eggs in most water-holding containers in a mosquito-infested area and consequently results in the emergence of adult mosquitoes unless the contents are poured out. In areas where there is frequent rainfall or irrigation, containers need to be emptied every 10 days (Lloyd et al. 2018). Dumping the contents does not remove eggs from the sides of the container. So, it should also be recommended that the container sides be scrubbed to dislodge and destroy eggs when dumping containers.

Despite most public health agencies recommending that people make every water-holding container a lethal ovitrap by dumping the contents, there is a lack of data showing that this method of control reduces adult mosquito populations. In low-income countries, water is often stored in small, uncovered containers around residences to serve as a source of potable water. These containers cannot be emptied without jeopardizing important water storage. Urban *Aedes* mosquitoes flourish in these conditions since there are many containers available for development. Even when communities are educated to empty containers that are holding water, those containers frequently are found to be producing mosquitoes, including important vectors of human disease (Marten *et al.* 2022).

Mass Ovi-trapping (MO). Ovitraps/cups were initially developed to detect the presence of *Ae. aegypti* during an eradication campaign of this mosquito in the Americas (Fay and Eliason 1966). Similar to the approach of surveillance, mass ovitrapping with dark cups has been used to harvest large numbers of mosquito eggs by inundating an area with oviposition cups. These cups were small dark containers that were usually black or red and made from plastic, metal, or glass. Water alone or water with decomposing organic material (water infusion) was added to each ovitrap. Mosquito eggs were collected on a substrate like a wooden tongue depressor or germination paper that would line the sides of the cup (Fig. 1). The substrate was collected, and the eggs were destroyed.

Figure 1. Ovicups are usually plastic drink cups with an oviposition surface attached to the side above water level.

Ovitraps target the destruction of eggs from ovipositing female mosquitoes by mass ovitrapping to ultimately reduce adult *Aedes* populations. A principal limitation of small containers is that frequent servicing of ovitraps is needed to replace water, attractants, and oviposition substrates. Servicing, besides destroying eggs, also prevents hatched larvae from becoming adults (Barrera *et al.* 2014a,b, 2019).

In practice, the ovicups (small-sized containers with less than 500 mL volume) were lined with an untreated oviposition surface, such as paper. After mosquitoes had a chance to oviposit, the papers or other surfaces were collected and destroyed. Regis *et al.* (2013) reported on mass-ovitrapping in Brazil where 8,400 2-L ovitraps were placed over a 2-year period in an integrated program by using top-feeding minnows, adult removal from buildings by aspiration, and public education. Over the 2-year period of the test, egg density was reduced by 90% in one community. Papers from the ovitraps were collected

and incinerated, destroying ~8 million eggs. By removing the egg stage from the wild mosquito population, the population of mosquitoes was sometimes reduced depending on the availability of alternative containers. Of course, when there were many alternative mosquito development sites in an area, this type of mass ovitrapping control did not show significant control effectiveness (Regis et al. 2013).

Lok Autocidal Ovitrap (LAO). The next stage in ovitrap development was a mechanical one, the Lok Autocidal Ovitrap (https://tougherthantom.com). This ovitrap was a mechanical device that prevented adult mosquitoes that developed inside the autocidal ovitrap, from escaping the container. The trap had a black plastic container for holding water, a plastic ring float sealed inside the hollow space of the container with fine netting in the center of the ring to prevent emerged mosquitoes from escaping. Two oviposition paddles were placed vertically on the top side of the float. Mosquitoes laid eggs on the paddles, and hatched larvae accessed the water beneath. Adult mosquitoes that emerged beneath the netting could not escape, so they died in the trap. The black LAO ovitrap was proven to be 81 to 97 times more attractive than natural containers (Lok et al. 1977).

This trap was used around the Singapore airport in the early 1970s for control of *Ae.aegypti* mosquitoes. *Aedes aegypti* mosquitoes were eradicated by mass-ovitrapping with the LAO ovitrap (Lok et al. 1977). The advantages of the trap were that it was not harmful to the environment, specific to urban *Aedes* mosquitoes, did not harm beneficial insects, did not contribute to environmental pollution or insecticide resistance, easy to use, economical for low-income countries, easily placed wherever mosquitoes are located, and did not require manpower for inspections and treatments (Lok et al. 1977). Despite all these advantages, the Lok ovitrap was susceptible to mechanical failure. This allowed mosquitoes to emerge from the traps and exacerbated the mosquito problem. Also, another issue with this trap was that the ovipositing female mosquitoes were not killed, so they were able to fly out of the trap where they entered.

Zeichner Lethal Ovitrap (ZLO). The Zeichner Lethal Ovitrap (https://www.inzecto.com) was an effective lethal ovitrap, which placed an insecticide-treated (pyrethroid) strip as an oviposition surface into a plastic cup. The ZLO addressed the shortcomings of space and residual insecticide applications by attracting the female mosquito to the insecticide treatment. Basically, the container used for the ZLO was a typical black plastic cup (473 ml) used by public health authorities for surveillance. The cup itself was inexpensive and well-known to be visited by urban *Aedes* mosquitoes. Gravid female mosquitoes found the walls of the cup to have too smooth of a surface to land and deposit their eggs, so they oriented to the pesticide-treated and rough-surfaced strip, which was preferred for mosquito landing and oviposition. The ZLO would kill the female mosquitoes and the larval mosquitoes that hatched through the insecticide strip contamination runoff into the water. The ZLO was patented by the U.S. Army and licensed to Springstar, Inc., which called it "Trap-N-Kill." The Trap-N-Kill innovation subsequently utilized a biodegradable plastic so the cup would degrade over time, not hold water long-term, and therefore, would not allow larval mosquito development once the pesticide was no longer effective. Another innovation in the ZLO was the addition of a hay and grass/water infusion that produced an odor that attracted ovipositing mosquitoes to the ZLO.

Figure 2. The Zeichner Lethal Ovitrap, a black plastic cup with a red strip of insecticide-treated oviposition paper attached. Overflow hole maintains water level.

The ZLO was evaluated in the field in Brazil, Peru, Bangladesh, and Thailand using 20 ZLOs per house, with 10 inside and 10 outside. Controls received no ovitraps. Traps were placed for three months, and *Ae. aegypti* populations were sampled each week. In all

four countries, the number of pupae (the most reliable measurement of mosquito populations) or containers with pupae decreased significantly. In the Thailand study, the reduction in mosquito population was less than in other countries and was due to the larger size and number of breeding containers, and possible immigration of mosquitoes from surrounding untreated areas (Zeichner and Debboun 2011, Sithiprasasna et al. 2003, Perich et al. 2003).

Most studies of the ZLO have involved an integrated vector management program with the application of biological controls, source reduction, and ZLO placement. In Australia, there were 87% fewer *Ae. aegypti* in treated areas compared with controls by the 4th week of treatment. In 2004, the use of 780 locally produced ZLOs treated with bifenthrin halted a dengue outbreak in Queensland (Rapley *et al.* 2009). The ZLO trap was concluded to be environmentally sound, economical, and a simple means of dengue and chikungunya vector control (Zeichner and Peritch 1999, Williams *et al.* 2007, Zeichner and Debboun 2011). The selection and application of the number of ovitraps per acre or per house follows the label and instructions.

An experimental fiber pot container and two other types of commercial ovitraps, empty containers (In2Care trap container only and SpringStar's TNK trap empty container only) after adding infusion water have been evaluated for the collection of *Aedes* eggs in northeastern Florida (Dixon et al. 2022). The results showed that the In2Care trap container only performed better than the fiber pot container and the TNK container.

Sticky Ovitrap (SO) and Autocidal Gravid Ovitrap (AGO). Sticky ovitraps just add glue to catch gravid females to kill. It is popular and easy to make. An additional step in ovitrap modification was the addition of glue on paper or substances. Several types of sticky ovitraps, such as the Sticky Ovitrap (SO) (https://tougherthantom.com) and the Autocidal Gravid Ovitrap (AGO) (https://catchmaster.com) have been developed that utilize glue to trap gravid female mosquitoes when they land to oviposit. These traps use glue above the water line in plastic buckets to capture ovipositing females. The advantage of capturing mosquitoes with glue is that insecticides are not used, there is no need to register the trap with a regulatory agency, and approvals and permits from countries are not needed.

The AGO trap is a large (19 L) black bucket with a 3.8 L black polyethylene cylinder entrance chamber that fits into the bucket lid. An adhesive panel covers the interior surface of the cylindrical entrance. The bottom bucket of the AGO is baited with 10 L of water and 30 g of hay as an infusion/water attractant. Adult mosquitoes are prevented from accessing water in the bucket with a screen that separates the entrance area from the water/infusion area (Mackay et al. 2013; Barrera et al. 2914a,b). AGO traps have been modified by adding a suction fan to increase the collection of gravid mosquitoes (Zhu et al. 2019). Also, combinations of attractants have been evaluated to attract and trap host-seeking mosquitoes not just ovipositing mosquitoes (Liu et al. 2019). In addition, the comparison of AGO traps and In2Care traps in St. Augustine did not show any significant difference in the collection of mosquitoes (Khater et al. 2022; Dixon et al. 2024). The evaluation of the AGO trap, CDC gravid trap (https://johnwhock.com), Biogents Gravid Aedes trap (GAT), and modified Biogent Bower (https://shop.biogents.com) were evaluated in northeastern Florida and demonstrated that the CDC gravid trap outperformed the others (Cilek et al. 2017 and 2017a; Xue et al. 2021). The SIRENIX (https://bentzjazusa.com) trap was compared against AGO traps in a field study in St. Augustine, Florida. The results demonstrated that the new lethal solar ovitrap (SIRENIX) outperformed by the AGO trap (Smoleroff et al. 2023).

The SO was similar but had a smaller design using a 1.2-liter black bucket with a plastic strip (21.5 × 5.5 cm) coated in polybutylene adhesive (UVR 32, Atlantic Paste and Glue, Brooklyn, NY) fastened to the inner wall of the bucket with large paper clips. The large size and addition of hay infusion to the SO and AGO traps increased mosquito collection efficiency when compared with the ZLO trap.

Figure 3. Sticky Ovitrap showing external and internal parts of the trap. The sticky paper is attached above the interior water line.

Another advantage of these glue traps is that mosquitoes entering the SO and AGO traps land on the sticky panel to lay eggs. Mosquitoes captured on the panel die and can be counted for surveillance. If enough traps are placed in an area, management is possible based on

the label and recommendation by the manufacturers. One problem associated with the SO and the AGO traps is that mosquito eggs can be washed through the screen into the water infusion. Those eggs can hatch and develop into adults. Even though the adults produced in the trap cannot escape, they can do so if the trap is opened or damaged. An additional study evaluating the addition of sticky paper inside the modified larval traps increased the catch rate of the emerging adult mosquitoes from the traps and reduced the escape of these emerged mosquitoes (Talbalaghi et al. 2020).

Two communities in San Juan, Puerto Rico were used to evaluate the operational problems of using AGO for mosquito control (Barrera et al. 2019). A total of ~18,000 traps were placed in the communities. Problems with the traps were observed for <2% of the traps (e.g., mosquitoes accessing the infusion, exclusion screen missing, sticky surface damaged/absent, trap missing). Immature and adult mosquitoes were found in the infusion for 7.5% and 9.1% of traps, respectively. Although specific for mosquitoes, lizards were found in 33% of the traps. Nontarget insects found in the glue were small flies and, more rarely, ants, cockroaches, grasshoppers, butterflies, and dragonflies.

Mass trapping with AGOs was highly successful in studies in Puerto Rico from 2011-2014. The first study involved about 1,000 traps and resulted in a 70% decrease in mosquitoes during the second year. The second study involved ~1,300 traps and resulted in an 88% reduction in mosquitoes during the second year (Barrera et al 2014a). Testing for Chikungunya virus exposure demonstrated ~50% reduction in people exposed to the virus (45.4% in the non-trap area with no traps vs 22.9% for areas with traps) (Barrera. 2014a,b). One major concern with these traps was that the glue was very effective in trapping and killing insectivorous reptiles, like geckos, that see trapped insects and enter the trap. The non-target trapping of insectivorous animals affects biological control organisms of many insect taxa (Ritchie *et al.* 2003, Ritchie, 2005, Ritchie *et al.* 2009, Chadee and Ritchie 2010, Gama *et al.* 2007). In a Florida study, 1,718 AGO traps were deployed in several subdivisions and demonstrated that the application of AGO traps reduced populations of *Aedes* mosquitoes; however, the results varied with different locations and seasonal changes (Dixon et al. 2024). Also, the infusion water and fermentation time in the lethal ovitraps impacted the collection of mosquitoes and non-target organisms (Mullin et al. 2020). The application of the number of AGO traps per acre or per house varied with the programs/ projects, locations, and budget.

Gravid *Aedes* Trap (GAT). The next invention was the Gravid *Aedes* Trap (GAT) (https://biogents.com). The GAT is a 1.2 L black plastic bucket with a matte finish that incorporates a black entry funnel (9.5 cm outer and 4.5 cm inner diameter, 8 cm high) to prevent mosquitoes from easily escaping the trap. The bucket contains a water infusion of vegetation to attract ovipositing mosquitoes to the odor of stagnant water. Above the water infusion mix, a black nylon screen mesh provides a barrier between ovipositing mosquitoes and the infusion. A translucent container, sprayed with a residual pesticide (e.g., pyrethroid), is inverted and placed between the bucket edge and funnel to provide a light tunnel. Mosquitoes attracted to the water infusion enter the trap through the funnel and are unable to pass through the screen to access the water infusion. They are attracted to the light coming through the translucent light tunnel, where they contact the residual insecticide and die as they rest on the surface. Because of the breakdown of the insecticide, it is recommended that the insecticide treatment be applied monthly to maintain efficacy.

Eiras et al. (2014, 2021; Heringer et al. 2016) evaluated the trap for mosquito knockdown and mortality and found that the residual pyrethroids provided 100% knockdown within 30 min. Ritchie *et al.* (2014) reported that the GAT is an improved lethal ovitrap because the killed mosquitoes can be counted for monitoring purposes and a variety of insecticide classes can be used to avoid resistance. The trap should be retreated monthly because the time to knockdown increased during the 8-week experiment. The trap can also be left untreated to allow the capture of live insects for virus or *Wolbachia* monitoring (Eiras et al. 2014). Eiras *et al.* (2021) found that GATs captured 50% - 65 % of mosquitoes regardless of the number and size of alternative breeding sites in the simulated field environment. A study by Figurskey *et al* (2022) documented that GATs would extend chemical control effectiveness when used in combination with barrier sprays at residences and that GATs resulted in a reduction of *Ae. albopictus* by 80.4% compared with untreated controls. There is a label and direction for the selection and application of the GAT.

Autodissemination Mosquito Trap (In2Care). The next phase in the evolution of ovitraps combines multiple strategies, including a fungal biological control. The In2Care trap (https://www.in2care.com) is composed of two stackable black buckets that hold a maximum of 3 L of water to allow long trap use and infrequent maintenance. The inner bucket holds the water that can overflow into the outer bucket. The outer bucket has three drainage holes to allow excess water to drain from the bottom. The lid has a funnel-shaped central opening that allows

rainwater to flow into the trap, allows mosquito entry and exit, allows return of evaporated/condensed water to the inner bucket, and also allows servicing of the trap. There is a floating ring in the center of the inner bucket that provides a landing and resting surface for mosquito oviposition. The floating ring holds an oviposition gauze that is treated with twoactive ingredients. The gauze is electrostatically dusted with 74% pyriproxyfen and an insecticidal fungus, *Beauveria bassiana* strain. When a mosquito lands on the gauze, the dust adheres to its body.

Snetselaar *et al.* (2014) showed that 90% of mosquitoes resting on the treated gauze of the In2Care trap were contaminated with insecticidal dust. The contaminated mosquitoes were able to leave the trap and visit other untreated containers to lay eggs. The surrounding containers were contaminated, and >90% of developing larvae were killed at pupation in the untreated breeding sites. Also, 100% of larvae developing in the trap itself were killed. In addition to the pyriproxyfen contamination for the larvae, the *B. bassiana* fungal spores contaminated adult mosquitoes (Sihuincha *et al.* 2005). The fungal spores provided a slow mode of action that killed female mosquitoes in 10-28 days. The directions recommend servicing the trap and replacing the gauze every 4-6 weeks.

Buckner *et al.* (2017) showed that untreated containers positioned ~2.8 m from the In2Care trap had 100% inhibition of adult mosquito emergence. Also, 80% of mosquitoes contaminated with the insecticidal gauze died within 10 days, compared with 41% in the untreated group. Buckner *et al.* (2021) did an extensive field evaluation of the In2Care trap, compared to an integrated pest management program. Traps used alone resulted in 60% fewer eggs, 57% fewer larvae, and 57% fewer adults, compared with the integrated vector management site. However, the authors discovered that traps may be less practical for control programs due to the time-consuming and labor-intensive work needed for trap deployment and maintenance. In comparing time, the integrated vector management program (ground and aerial ULV, larvicide application and source reduction, and citizen service requests) took ~156 hours, whereas the In2Care trap installation and maintenance involved 780 manhours, or about 5x more time for a modest reduction in mosquito numbers. Buckner et al (2025) reported that a completed trial in 2024 demonstrated that In2Care might also aid in the reduction of *Culex* mosquitoes.

According to the manufacturer labeling, the autocidal control involves using female mosquitoes as vehicles for transporting pyriproxyfen to breeding sites and reducing larval populations by allowing females to land on treated netting, picking up the larvicide and the fungus. The mosquitoes then distribute the insecticidal products to other water containers due to their propensity to distribute their eggs through skip oviposition into different containers. While doing so, the treated females transfer pyriproxyfen and the fungal spores, which, in very small doses, are lethal to larvae and pupae (Itoh et al. 1994, Ali et al. 1995, Nayar et al. 2002, Sihunicha 2005, Devine et al. 2009). Before taking another blood meal, the females exposed to the fungal spores die, eliminating them as disease vectors (Clark et al. 1968). The In2Care trap label suggests avoiding prolonged or repeated exposure to microbial proteins found in the *Beauveria s*pores due to the potential development of allergic sensitization. To prevent that exposure, the label requires the use of a particulate respirator when handling the treated gauze strip.

Laboratory and semi-field trials have demonstrated a decreased vectorial capacity in trap-exposed mosquito females, with reduced feeding behaviors and increased mortality (Blanford et al. 2011, Darbro et al. 2012). The In2Care traps appear attractive to *Aedes*, with successful pyriproxyfen dispersal and high larval mortality to nearby breeding sites, as well as high adult mortality from *B. bassiana* infection. Besides *Aedes* mosquitoes, In2Care traps can attract and kill *Culex quinquefasciatus Say* (Su et al. 2020). However, another study in the USA found that autodissemination of pesticides on *Ae. albopictus* did not produce positive results (Unlu et al. 2020). There are several other studies that have attempted to study the impact of autodissemination on mosquito populations and comparison with other lethal ovitraps in the field, but the results did not show significant differences (Autry et al. 2021; Khater et al. 2022). The applications needs to be following the label and instructions due to relate to insecticides.

Dual-Action Lethal Ovitrap (Inzecto Mosquito Trap). As detailed in this article, several versions of lethal ovitraps have shown the potential to kill container mosquitoes, such as urban *Aedes*. The ovitraps compete with other containers for mosquito oviposition sites because other containers can even include abandoned swimming pools, fountains, cisterns, discarded items, and small bodies of water (Fischer and Schweigmann 2010). Lethal ovitraps kill mosquitoes when they come to lay eggs in a container. The problem with single-action lethal ovitraps is that the mosquitoes often lay their eggs before they die. Those eggs then hatch, and the container allows for larval development and adult mosquito emergence. The dual-action lethal ovitrap, currently sold under the trade name Inzecto Mosquito Trap (https://www.inzecto.com), contains both an adulticide (permethrin)

and an insect growth regulator, pyriproxyfen. With this dual-action strategy, two mosquito life stages, adults and pupae, are targeted within the same container (Parker et al. 2017), without reliance on the autdissemination of the insecticides. The expectation is that any female depositing eggs within the trap will be killed by the adulticide, and because of the insect growth regulator, no progeny of that female will develop into adults that could vector diseases.

Once the elements were in place to entice the female mosquitoes to visit the ovitrap and deposit their eggs, two other elements were necessary to complete this complex environment in order to maximize its mosquito killing capabilities: a) an adulticide, permethrin, that would kill mosquitoes resting on the interior surface and prevent the female from ovipositing again in other containers, and b) a larvicide, pyriproxyfen, that would prevent the development of biting adult females. To delay the breakdown of these chemicals by hydrolysis, the inside of the trap is coated with a long-lasting, time-released polymer that contains the adulticide and larvicide. A unique innovation of this trap is that the polymer also contains a texturizing agent that enhances the time-release activity so the trap continues to release the active ingredients continually over three months. This coating is located on the inside surface of the trap, preventing humans and animals from contacting the active ingredients. The trap is activated by the addition of 300 mL of water. The internal long-lasting insecticidal texturized coating and dry space in the ridges allow for adult mosquitoes to land inside the trap, above the water, to be exposed to the treatment. The coating throughout the trap also allows for any larvae that emerge from eggs laid at the edge of the water to develop normally until the last stages of development when the effect of the larvicide, pyriproxyfen, prevents further development and emergence of new adults (Dhang 2023). Khater et al. (2019) demonstrated in a semi-field evaluation that modifying this trap by adding stick paper inside the trap increased the collection of gravid female mosquitoes.

In contrast with standard ovitraps whose tops are open or mostly open, the Inzecto Mosquito Trap was designed with a lid to protect from quick evaporation, direct sunlight, wind, and rain so that liquid in the base would be available for larval exposure to the insect growth regulator that prevents pupation. To further enhance the attractiveness that allows this trap to outcompete other water sources for mosquito oviposition, the contrasting mosquito-attractive colors (red and black) are incorporated. The ridges provide high-humidity dead air space that mosquitoes need for survival. This also increases the surface area for egg laying. A leaf-filled sachet is included to provide an attractive infusion in the water (Browne & Bennett 1981). Parker *et al.* (2017) documented a 94% reduction in urban *Aedes* eggs laid in nearby containers when an Inzecto ovitrap was placed in the proximity of other typical containers found in the urban landscape.

The dual-action lethal ovitrap was financed by the Armed Forces Pest Management Board through the Deployed Warfighter Protection program made available for the development of novel concepts in the control of mosquitoes, with emphasis on technologies. This kind of trap could be easily deployed by the Armed Forces in combat zones with minimal to no advanced training. Requirements for a technology to be used in combat areas include portability, being ready to deploy, and simplicity of use. The qualities are also desirable for commercial products that can be used by professional pest control operators and consumers with varying levels of understanding of the mosquito life cycle, developmental needs, and environmental requirements.

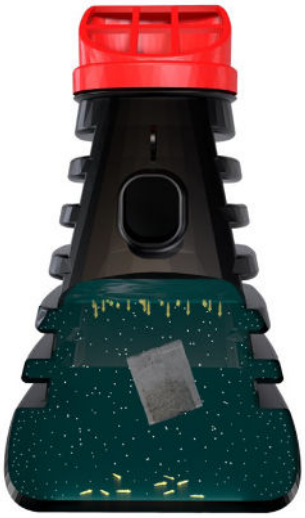

Figure 4. Inzecto dual-action lethal ovitrap with ridges, contrasting colors, evaporation protection, and overflow spout. The dual-action permethrin and pyriproxyfen in a polymer coating is internal, as is the attractive sachet. The application and selection should follow the trap label and operation instruction.

The efficacy of the dual-action lethal ovitrap relies on eliminating the present and the future generation of mosquitoes with minimum environmental contamination and maximum efficacy. The Inzecto trap has been approved in 23 countries worldwide.

Summary & Future of Lethal Ovitraps. The application of lethal ovitraps is a part of integrated mosquito management. There are several types of lethal

ovitraps on the market and many influencing factors on the selection and amount of traps used per acre or per house, such as program budget, labor, location, target species and objective, type and size of the traps, label and instruction of each product, and the residents' acceptance. Also, the label and instruction reading are required for the selection and use of any kind of lethal ovitraps combined with insecticides. There are always new developments and new discoveries that can be aggregated to old ideas in the formulation of a new line of research and product development. Society challenges scientists and entrepreneurs to further their knowledge and understanding of the world and the products created, especially those used against mosquitoes. The attraction of the infusion for the gravid female mosquitoes, the combination of attractants and trap design for gravid and host-seeking female mosquitoes and male mosquitoes, and the active ingredient of contact poison or sticky are still underexplored and further research and development is needed.

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CULEX (PHENACOMYIA) LACTATOR, A NEW MOSQUITO SPECIES IN BROWARD COUNTY, FLORIDA

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ABSTRACT

Culex (Phenacomyia) lactator (Diptera: Culicidae) was detected in Broward County. Here we describe a single larval habitat where *Cx. lactator* larvae were collected in the winter of 2023 and 2024 in the City of Miramar. Adult specimens of this species were collected from two locations in fall 2022 using CDC light traps and in winter 2023 using Biogents BG-Sentinel 2 traps. In this paper, we provide larval habitat description and photographic evidence of morphology consistent with *Cx. lactator* larvae and adults.

Key words: mosquito, *Culex lactator*, non-native, invasive, county record, morphology, taxonomy

INTRODUCTION

Culex (Phenacomyia) lactator (Diptera: Culicidae) Dyar & Knab, 1906, is one of three species of the *Phenacomyia* subgenus first described by Harbach and Peyton (1992). Few studies have been published on *Phenacomyia* exacerbated by unresolved systematics (Belkin et al. 1970) and presumed undescribed species with adults morphologically similar to *Culex corniger* and *Cx. lactator* (Strickman & Pratt 1989). Furthermore, a lack of robust sampling and vector competency studies makes it difficult to evaluate the potential role of the subgenus *Phenacomyia* as a vector for pathogens. For instance, blood meal analyses indicate potential avian preference; *Cx. lactator* blood meals recovered in Guatemala were chicken (n=1, *Gallus spp.*) and red-winged blackbird (n=1, *Agelaius phoeniceus*) (Kading et al. 2013), one *Cx. corniger* blood meal collected in Chiapas State, Mexico was chicken (n=1) (Hernández-Triana et al. 2020), and one blood meal collected in Miami-Dade County was a passerine bird (n=1) (Reeves et al. 2023). In contrast, a single pool of *Cx. lactator* collected in Xmatkuil, Yucatán, Mexico tested positive for Zika virus (Nunez-Avellaneda et al. 2021). Moreover, parenterally inoculated *Cx. corniger*, were capable of transmitting Maguari virus (*Orthobunyavirus maguariense*) to mice in the first 7-23 days of life (CDC 2024).

Phenacomyia mosquitoes have been recorded as far north as the Caribbean region and Mexico, e.g., *Cx. corniger*, and as far south as Uruguay and Argentina, e.g., *Culex (Phenacomyia) airozai*, *Cx. corniger* (Perez Vigueras 1956, Knight & Stone 1977, Rossi 1996, Broche 2008, Ortega-Morales et al. 2021). Little is known about *Cx. lactator*, but collection records are limited to central Mexico and northern South America (Colombia) with no collections in the Caribbean region (Strickman and Pratt 1989). Strickman and Pratt (1989) described the distribution of *Cx. lactator* occurring near sea level and as much as 1,500 m above sea level. Aquatic larval habitats include sunlit ground pools, streams or lake margins, and containers filled with decaying vegetation (Strickman and Pratt 1989, Baak-Baak et al. 2016). Recently, *Cx. lactator* has been reported in south Florida, representing the first record of this Neotropical subgenus in the United States (Reeves et al. 2023). It was first detected in southern Miami-Dade County in 2018 and later in Collier and Lee counties in 2022 (Reeves et al. 2023). *Culex lactator* was not collected in 2023 in Lee County Mosquito & Hyacinth Control Districts (A. Loyd, personal communication, 13 May 2024) but was collected in 2023 by the Miami-Dade Mosquito Control Division (C. Vasquez, personal communication, 8 May 2024) and in 2023 and 2024 by the Collier Mosquito Control District (K.J. Lucas, personal communication, 8

May 2024) (Table 1). Other collections include Palm Beach County Division of Mosquito Control in 2023 (S. Fazekas, personal communication, 21 May 2024). No collections were made in 2021. To date, the Florida Keys Mosquito Control District has not detected *Cx. lactator* in Monroe County (A. Leal, personal communication, 21 May 2024).

Table 1. Updated collection records for *Cx. lactator* in Florida, USA (2018 – 2024). ASP = aspirator; BG = Biogents Sentinel Trap; CDC = CDC Miniature Light Trap; Dipper = mosquito sampling dipper for larvae and pupae; DF = mosquito drift fence; GRA = gravid trap. Data was obtained from Reeves et al. (2023) and personal communications from mosquito control districts.

Year	County	Life Stage (N)	Collection Method (N)
2018	Miami-Dade	adults (4)	CDC (4)
2019	Miami-Dade	adults (63), larvae (5)	CDC (17), BG (1), Dipper (5), DF (40), ASP (5)
2020	Miami-Dade	adults (13)	ASP (3), CDC (2), DF (8)
2022	Broward	adult (1)	CDC (1)
2022	Lee	larvae (47)	Dipper (47)
2022	Collier	adults (67)	BG (6), CDC (33), GRA (28)
2022	Miami-Dade	adults (10)	CDC (10)
2023	Broward	adults (3), larvae (4)	BG (4), Dipper (3)
2023	Collier	adults (17)	CDC (17)
2023	Miami-Dade	adults (295), larvae (2)	BG (2), CDC (293), Dipper (2)
2023	Palm Beach	adults (6)	CDC (6)
2024	Collier	adults (4)	CDC (4)

More recently, in Broward County, we identified larval and adult *Cx. lactator* from three collection sites separated by 6.5, 30.4, and 34.0 km of urbanized landscape (Table 2). Staff members D. Duguma and S. Garcia from the Broward County Mosquito Control Section collected *Cx. lactator* larvae with a standard mosquito sampling dipper (John W. Hock Company, Gainesville, FL) on 11 January 2023, in an area in the southwest of the county (Lat. 25.98742, Lon. -80.40895), in the City of Miramar 6.5 kilometers west of the light trap collection site. On 12 January 2023, D. Duguma, S. Garcia, and E. Miqueli collected three additional larvae from the same collection site (Fig.1). Larvae were collected on the ground in a remnant of a flooded area about to dry up and associated with basket grass, *Schoenoplectus pungens (Vahl)* (Fig.1). *Culex lactator* were found coexisting with *Culex (Culex) nigripalpus* Theobald, 1901 and *Culex (Melanoconion) erraticus* (Dyar & Knab, 1906) at the same collection site, suggesting a potential shared niche during the larval stage.

Table 2. Collection records for *Cx. lactator* in Broward County, FL USA (2023 – 2024). BG = Biogents Sentinel 2 Trap; CDC = CDC Miniature Light Trap; dipper = mosquito sampling dipper for larvae and pupae. All adult traps were baited with 1.5 kg of CO_2. Adult collections were host-seeking females. Parity was not assessed.

Date	City	Lat, Long	Number/Life Stage	Collection Method
9/14/2022*	Miramar	25.98570, -80.34420	1/adult ♀	CDC
1/11/2023	Miramar	25.98742, -80.40895	1/larva ♂	Dipper
1/12/2023	Miramar	25.98742, -80.40895	3/larva ♀	Dipper
1/24/2023	Margate	26.22373, -80.19261	3/adult ♀	BG

*Sample conserved in freezer (-20°C) and processed after larval samples were collected and identified

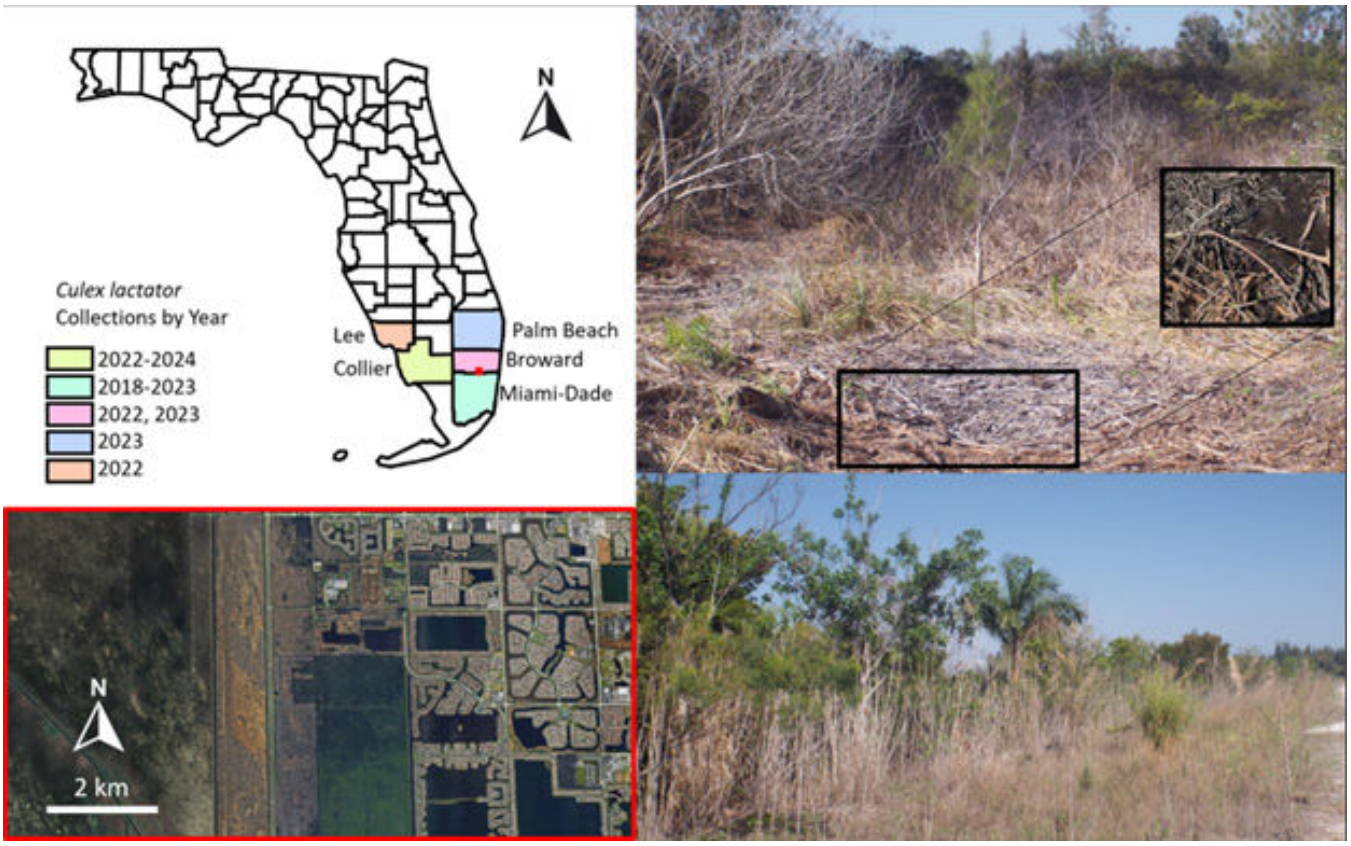

Figure 1. County-level distribution of *Culex lactator* collections in Florida 2018 to 2024, satellite imagery of portions of Miramar to highlight proximity to Florida Everglades, and photographs of a small pool and vegetation surrounding the larval collection site where *Cx. lactator* larvae were collected on 10 January 2023. Latitude: 25.98742, Longitude: -80.40895.

The discovery of *Cx. lactator* larvae prompted B. Giordano and E. Miqueli to revisit trap collections from the surrounding area. A single specimen was collected from a dry-ice baited CDC Miniature Light Trap (Model 512, John W. Hock, Gainesville, FL) set on 14 September 2022, in the City of Miramar (Lat. 25.985700, Lon. -80.344160). The CDC trap was set adjacent to a storm water management pond that supports *Cx. erraticus*, *Mansonia (Mansonia) dyari* (Dyar & Knab, 1906), and *Mansonia (Mansonia) titillans* (Walker, 1848) production. On January 24, 2023, we collected three specimens of *Cx. lactator* in a Biogents Sentinel 2 trap (BG; Biogents AG, Regensburg, Germany), baited with 1.5 kg of dry ice, in the City of Margate (Lat. 26.22373, Lon. -80.19261) 34.0 km north-northeast of the locality where we collected the larvae. The trap was set along the edge of Fern Forest Nature Center. This designated urban wilderness area covers approximately 1 km². It presents a variety of ecosystems: hardwood forests, prairies, hammocks, and pineland communities, is inhabited by more than 200 plants species, and is home to several species of mammals, reptilians, birds, mollusks, and arthropods (https://www.broward.org/Parks/Pages/park.aspx?park=14). No further collections have been made.

We placed collected larvae (n=4) in an emerging cup filled with rainwater at room temperature and in approximately 38 hours observed pupation. Of these, a total of three females and one male emerged between 56 to 96 hours after pupation. Both the exuviae of the larvae and the pupae, the genitalia of the male, and the front leg of each adult were conserved in 95% ethanol for future study. All the adult specimens were mounted on micro pins for photography using a microscope digital camera (Model MU1403, AmScope, Irving, CA) (Fig.2). Mosquito species identification was performed by E. Miqueli and B. Giordano using Bonne & Bonne-Wepster (1925) [for *Culex corniger*], Strickman & Pratt (1989), Harbach & Peyton (1992), and later confirmation with L. Reeves (University of Florida). *Culex lactator* adults have a brown and drab appearance with the following notable ornamentation: scutum characteristically bordered with gold scales, abdomen with lightly colored basal bands, anterior median area of vertex with narrow gold scales, thorax showing three clusters of white scales surrounding

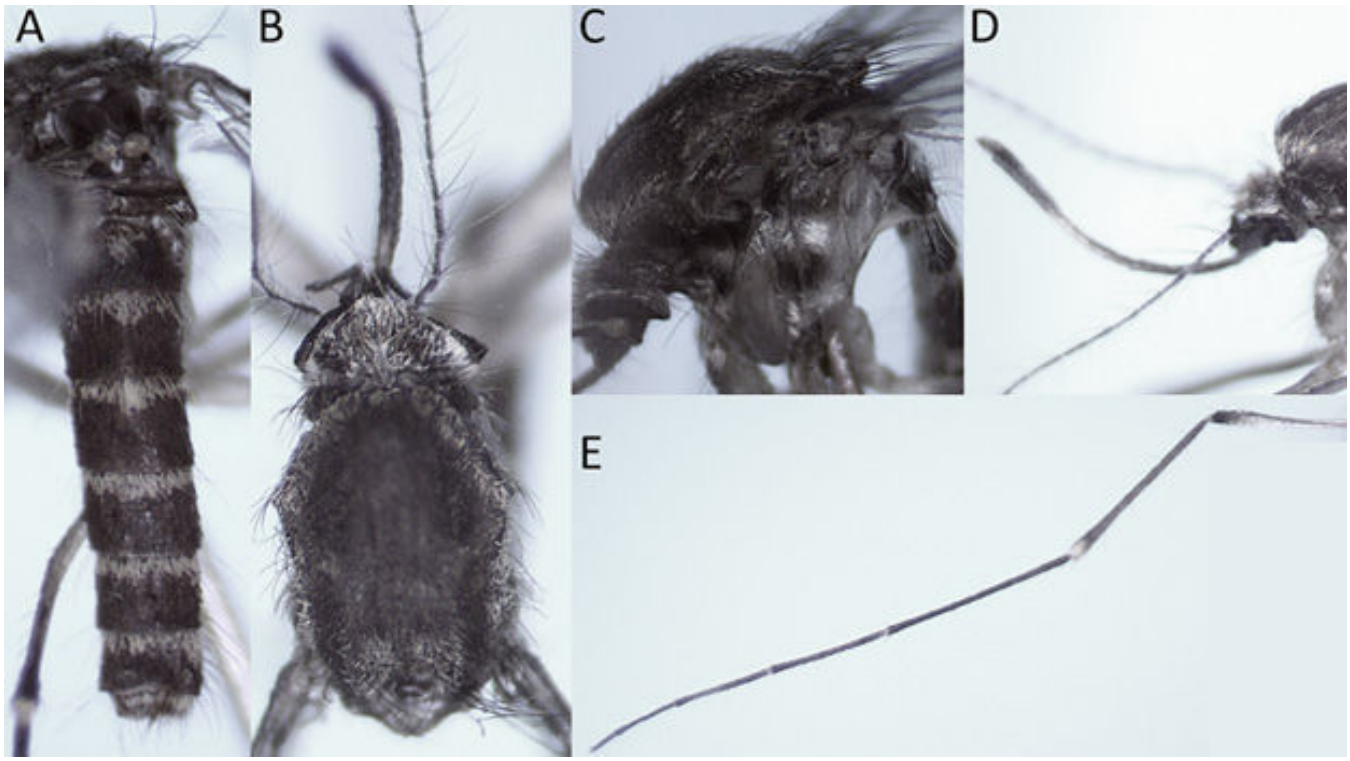

Figure 2. Key morphology for the identification of adult female *Cx. lactator.* A: Abdomen with lightly colored basal bands. B: Scutum and occiput. Scutum is characteristically bordered with gold scales. The anterior median area of the vertex with narrow gold scales; the patch of broad white scales is present laterally. C: Lateral view of thorax showing three clusters of white scales surrounding a circular patch of dark integument. D: Proboscis showing pale-scaled incomplete band. The proboscis is shorter than the antennae. E: Female hind leg with narrow basal and apical bands (faint) on tarsomere 1.

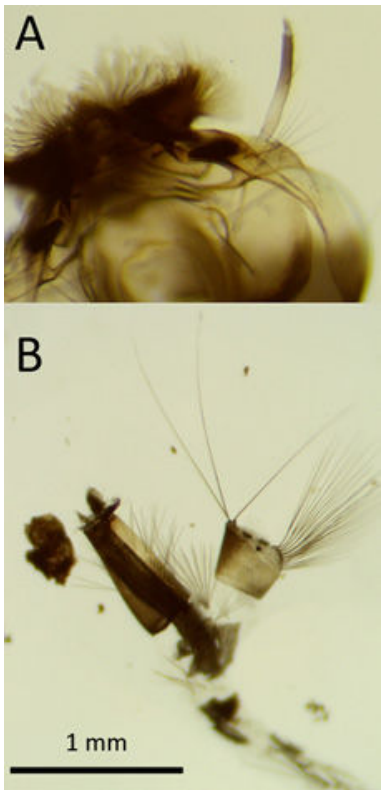

Figure 3. Key morphology for the identification of *Cx. lactator* larva. A: Head and antenna showing characteristically uniform, not obviously tapered apically, antenna. B. Saddle and siphon showing short and stout siphon with long branched setae beginning at mid-length.

circular patch of dark integument, proboscis (shorter than antennae) showing pale-scaled incomplete band (Fig.2). *Culex lactator* larvae have a short and stout siphon (siphon index ~2) with prominent branched setae beginning at mid-length and short uniform antennae (Strickman & Pratt, 1989) (Fig 3).

The discovery of *Cx. lactator* in Broward County is part of an upward trend of Neotropical mosquito species becoming established in the state of Florida. Over the past decade *Aedes* (*Ochlerotatus*) *pertinax* (Shroyer et al. 2015), *Aedes* (*Ochlerotatus*) *scapularis* (Reeves et al. 2021), *Culex* (*Culex*) *declarator* (Darsie and Shroyer 2004), *Culex* (*Culex*) *coronator* (Connelly et al. 2016), and *Culex* (*Culex*) *interrogator* (Shin et al. 2016) have become established here in Broward County (unpublished data , Broward County Mosquito Control Section). For example, *Ae. pertinax, Ae. scapularis,* and *Cx. coronator* are now abundant species collected in light traps following heavy rainfall events (unpublished data, Broward County Mosquito Control Section). With many recent non-native mosquito introductions over a short period of time, regional morphological identification keys become outdated, often missing recent species records or distributions. New

species introductions can be missed if mosquito control taxonomists are not aware of species distributions in adjacent counties, states, and regions (e.g., the Caribbean region, Central and South America) and current published literature. The use of multiple resources for mosquito identification is standard practice given South Florida's proximity to Central and South America, shipping yards/ports, and international airports. Therefore, mosquito taxonomists must have the most updated information available.

Here we describe a single larval habitat where *Cx. lactator* was discovered in low abundance, the surrounding environment of adult collections, and provided photographic evidence of morphological characters consistent with *Cx. lactator*. Repeated collections across all life stages, the distance between the locations, the short time elapsed between the respective detections, and evidence of *Cx. lactator* in adjacent and nearby counties (Reeves et al. 2023), suggests that this species may be more widespread in Broward County. At this time, we cannot speculate how the presence of *Cx. lactator* will affect disease transmission or nuisance complaints in Broward County. Point of introduction, host preference, larval habitat, and involvement in disease transmission cycles in Broward County remain to be elucidated for *Cx. lactator*. It is currently unknown how the introduction of this Neotropical species will affect mosquito control operations in the county until more information about its ecology and seasonal phenology of this species is known. We hope reports such as these raise awareness of non-native mosquito species detection in south and central Florida.

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EVALUATION OF A CHEMICAL BLEND TO ATTRACT THE ADULT MOSQUITO *AEDES AEGYPTI* (DIPTERA: CULICIDAE) BY AN OLFACTOMETER

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ABSTRACT

This study aimed to develop and evaluate synthetic chemical blends designed to attract *Aedes aegypti* for use in mosquito monitoring and integrated pest management programs. Laboratory experiments were conducted using a dual-port olfactometer to assess the attractiveness of various chemical blends composed of acetone, lactic acid, octenol, hexanoic acid, and other additives, including cyclopentanone, ammonium bicarbonate, linalool oxide, and beetroot-based sucrose solutions. The results identified a blend of acetone, lactic acid, octenol, and hexanoic acid (Solution 1) as a highly effective attractant, achieving a 61.8% attraction rate, significantly outperforming a lower-concentration variant ($p=0.047$) and the commercially available BG lure ($p=0.033$). Additives such as cyclopentanone and ammonium bicarbonate significantly enhanced mosquito attraction (67.8%, $p=0.012$), as did linalool oxide (68.5%, $p=0.007$), suggesting synergistic effects with the base blend. However, other additives, such as beetroot-based sucrose solutions, showed limited efficacy.

This study highlights the potential of optimized chemical blends to improve mosquito surveillance tools and enhance vector control strategies by targeting and concentrating *Ae. aegypti* populations. The findings pave the way for further field validation and integration into existing mosquito management programs, offering a promising approach to reducing the burden of mosquito-borne diseases.

Key words: host-seeking behavior, chemo-ecology, surveillance, control

INTRODUCTION

Mosquitoes are among the most important vectors of infectious diseases worldwide, transmitting pathogens that cause malaria, dengue, chikungunya, Zika, and various other illnesses. These diseases result in significant morbidity and mortality, particularly in tropical and subtropical regions. The ability to monitor and control mosquito populations is crucial for reducing the transmission of these diseases and mitigating their public health impact. Traditional methods of mosquito control and surveillance often rely on insecticides, physical traps, and repellents (Barreaux et al., 2017; Li et al., 2016; Xue et al. 2012; Montenegro-Quiñonez et al., 2023; Kumar et al., 2024). While these approaches have been effective to a degree, they face challenges such as developing insecticide resistance in mosquito populations and accurately assessing mosquito densities in diverse environments (van den Berg et al., 2021). Recently, there has been a growing interest in using chemical attractants as a more targeted and effective approach to mosquito surveillance and control.

Mosquitoes are naturally drawn to their hosts by a variety of cues, including body heat, visual stimuli, and most notably, olfactory signals. Studies have shown that mosquitoes use the carbon dioxide (CO_2) exhaled by humans, alongside volatile organic compounds (VOCs) emitted from human skin, such as lactic acid, ammonia, and certain fatty acids, to locate and approach their hosts (De Obaldia. et al., 2022; Wooding et al., 2020). These cues are highly attractive to mosquitoes and can be mimicked in the laboratory to create chemical blends designed to lure mosquitoes into traps or specific areas for population monitoring and reduction. The development of an effective chemical blend for mosquito attraction involves the identification of key compounds that mimic these natural cues and their formulation into a stable, reliable product that can be used in the field. By developing such blends, researchers aim to improve the efficiency of mosquito traps and provide more accurate data on mosquito population dynamics (Spanoudis et al., 2022; Xie et al., 2019). These chemical attractants can also be integrated into vector control programs as part of an integrated pest management (IPM) approach, enhancing the efficacy of existing tools by concentrating mosquito populations in target areas for more efficient intervention.

The objective of this study was to evaluate the attractiveness of various chemical blends to *Aedes aegypti* (Linnaeus) mosquitoes using a dual-port olfactometer. By comparing the response of mosquitoes to different formulations, including those containing acetone, lactic acid, octenol, hexanoic acid, cyclopentanone, ammonium bicarbonate, and beetroot extract, the study sought to determine the optimal blend that could enhance vector monitoring strategies. The goal is to provide a novel tool for mosquito surveillance and control, potentially improving the management of mosquito-borne diseases.

MATERIALS AND METHODS

Laboratory experiments were conducted to evaluate the effectiveness of the chemical blends in attracting mosquitoes under controlled conditions. The following procedure was followed:

Chemical Selection Criteria: The chemicals selected for the blend were based on their proven efficacy in attracting mosquitoes and their ability to mimic natural host odors. The goal was to create a balanced blend that combines attractants for maximum effectiveness. The chemicals selected (Table 1) were i) acetone, ii) lactic acid, iii) octenol, iv) hexanoic acid, v) nonalal, vi) cyclopentanone, vii) linalool oxide, viii) ammonium bicarbonate, and ix) BG lure. Mixtures of different chemicals were prepared as solutions 1-6 and 8-13 as listed in Table 1.

Mosquito Species: The mosquito species selected for testing included *Ae. aegypti* (dengue and Zika vector), as they represent one of the most medically important mosquito vectors. Mosquitoes (preferably 3-5 days old) were 2016 St. Augustine strain reared at Anastasia Mosquito Control District (AMCD) insectaries maintaining temperature at 80±3°F, relative humidity at 75±10%, and a photoperiod of 14L:10D.

Experimental Procedure: A true choice olfactometer (Sigma Scientific, Micanopy, FL) at the AMCD, was used for the assessment of the attractiveness of the chemical blends following Farooq et al. (2022). Briefly, the olfactometer consisted of two choice chambers (front and rear), a mosquito release chamber, two odor release chambers, and flow control valves. Pairs of two of the 12 solutions were compared with each other as listed in Table 2 to make tests 1-10. For each test, one each of the two solutions being tested was placed in the front or rear odor release chambers. The flow of clean and dry air was maintained at 6.8 liters/min for each odor-release chamber and 3.6 liters/min for the mosquito-release chamber. For each experimental run, airflow was turned on, and 24-hour-starved *Ae. aegypti* mosquitoes (15–30 individuals) with access to water only were introduced into the mosquito release chamber. Mosquitoes were allowed to acclimatize for 30 minutes before the start of the experiment. After 15 minutes of release, the number of mosquitoes in the front choice chamber, rear choice chamber, and mosquito release chamber was counted and

Table 1. The chemical blends were selected and used for the experiment.

Name	Chemicals used
Solution 1	acetone (4.1 ml) + lactic acid (500 µl) + octenol (200 µl) + hexanoic acid (200 µl)
Solution 2	acetone (4.72 ml) + lactic acid (200 µl) + octenol (40 µl) + hexanoic acid (40 µl)
Solution 3	solution 1 (980 µl) + nonalal (20 µl)
Solution 4	solution1 (980 µl) + linalool oxide (20 µl)
Solution 6	acetone (3.8 ml) + lactic acid (500 µl) + octenol (200 µl) + hexanoic acid (200 µl) + cyclopentanone (300 µl)
Solution 8	acetone (1 ml) + lactic acid (500 µl) + octenol (200 µl) + hexanoic acid (200 µl) +cyclopentanone (300 µl) + 10% ammonium bi carbonate in water (2.8 ml)
Solution 9	acetone (1 ml) + lactic acid (1 ml) + octenol (200 µl) + hexanoic acid (200 µl) +cyclopentanone (300 µl) + 10% ammonium bi carbonate in water (2.3 ml)
Solution 10	lactic acid (500 µl) + octenol (200 µl) +hexanoic acid (200 µl) +10% ammonium bi carbonate in water (4.1 ml)
Solution 11	lactic acid (500 µl) +octenol (200 µl) +hexanoic acid (200 µl) +10% ammonium bi carbonate in water (3.6 ml) +acetone (1 ml)
Solution 12	acetone (1 ml) +lactic acid (1 ml) +octenol (200 µl) +hexanoic acid (200 µl) +cyclopentanone (300 µl) +10% ammonium bi carbonate in beet root with sucrose (2.3 ml)
Solution 13	beet root in sucrose (1:1) +BG lure (3%)

Table 2. Comparative attraction of *Aedes aegypti* for different chemical blends using a dual port olfactometer

Test	Treatments	% attraction*	P value
1	Solution 1	61.8±7.1[a]	*p=0.047*
	Solution 2	38.2±7.1[b]	
2	Solution 3	31.5±7.3[b]	*p=0.007*
	Solution 4	68.5±7.3[a]	
3	Solution 1	56.4±5.0[a]	*p=0.110*
	Solution 6	43.6±5.0[a]	
4	Solution 1	53.4±9.4[a]	*p=0.625*
	Solution 11	46.6±9.4[a]	
5	Solution 1	45.1±6.3[a]	*p=0.307*
	Solution 10	54.9±6.3[a]	
6	Solution 1	32.2±7.7[b]	*p=0.012*
	Solution 9	67.8±7.7[a]	
7	Solution 1	55.5±3.0[a]	*p=0.033*
	BG lure	44.5±3.0[b]	
8	BG lure	41.3±5.9[a]	*p=0.071*
	Solution 9	58.7±5.9[a]	
9	Solution 8	47.0±7.2[a]	*p=0.574*
	Solution 9	53.0±7.2[a]	
10	Solution 12	45.7±9.2[a]	*p=0.532*
	Solution 13	54.3±9.2[a]	

*Means with same letters within a column are not significantly different at 95% confidence.

recorded. Between runs, all mosquitoes were removed from the olfactometer to prepare for the next replication. Each treatment was replicated five times. After completion of the test, solutions were removed, and clean air was run through the system for 30 minutes to clean any vapors before the next test. The number of mosquitoes in each chamber was expressed as a percentage of the total mosquitoes used per run. These percentages were used to calculate mosquito activation and attraction to different chemical blends. Activation was defined as the percentage of mosquitoes that exited the release chamber to either of the odor release chambers.

Data Analysis: All data analyses were performed using the statistical software JMP (version 15.2). The results were analyzed by calculating the percentage of mosquitoes attracted to each blend relative to the comparative blend. Statistical significance was determined using a paired t-test to assess whether the differences in attraction rates were significant. The activation data for all tests found being non-normal was analyzed using the nonparametric Wilcoxon test to assess the significance of the effect of pairing on mosquito activity at 0.05 level of significance utilizing JMP version 15.2.0 (SAS Institute, Cary NC). The means comparison was done using Wilcoxon each pair test of nonparametric analysis.

RESULTS

The mosquito activity represented by the activation percentage during the evaluation of various pairs of solutions was affected by the pair used in testing ($\chi^2 = 18.6$, $df = 9$, $p = 0.028$). The means comparison revealed that tests 1, 3, 4, and 7 had significantly higher activity than tests 2 and 5. The tests 6, 8, 9, and 10 did not separate from the other two groups of tests.

A blend of acetone (4.1 ml) +lactic acid (500 µl) +octenol (200 µl) +hexanoic acid (200 µl) showed significant attraction (61.8%) as compared to the blend with a lower concentration of lactic acid, octenol, and hexanoic acid (solution 2) (*p=0.047*). The same blend was also more attractive (55.5%) as compared to BG lure (44.5%), and the difference was significant (*p=0.033*). The addition of both cyclopentanone and ammonium bicarbonate solution to the chemical blend of acetone (1 ml) +lactic acid (1 ml) +octenol (200 µl) +hexanoic acid (200 µl) significantly attracted (*p=0.012*) more mosquitoes (67.8%). Further, lowering the concentration of lactic acid lowered the attraction rate, but it was non-significant. The addition of linalool oxide to the blend of acetone (4.1 ml) +lactic acid (500 µl) +octenol (200 µl) +hexanoic acid (200 µl) attracted *Ae. aegypti* significantly (*p=0.007*)

as compared to the addition of nonalal to this blend (Table 2).

DISCUSSION

Olfactometer experiments with different chemical blends showed that solution 9, having a mixture of acetone (1 ml) + lactic acid (1ml) + octenol (200 µl) + hexanoic acid (200 µl) +cyclopentanone (300 µl) + 10% ammonium bicarbonate in water (2.3 ml) seems to attract most the *Ae. aegypti* mosquitoes, followed by solution 1 consisting of acetone (4.1 ml) +lactic acid (500 µl) +octenol (200 µl) +hexanoic acid (200 µl). These two chemical blends were also found to act better than the BG lure. However, a combination of chemical blend with beetroot juice (solution 12) was not as effective as BG lure in beet root juice (solution 13). The results indicate that the developed chemical blend might effectively mimic the natural host cues, enhancing the trapping efficiency of mosquito traps. This development has potential applications in mosquito surveillance programs and integrated pest management (IPM) strategies.

It is well known that chemical blends from human skin volatiles attract mosquitoes (Hansonn et al., 2011; De Obaldia et al., 2022). Several studies have been undertaken in this quest for the development of an effective chemical blend (Wooding et al., 2020). BG traps were developed for trapping *Ae. aegypti* mosquitoes also consist of BG-Lure having a blend of lactic acid, ammonia, and hexanoic acid (Xie et al., 2019). However, due to the variety of olfactory signals to locate the host, BG lure is not the most efficient attractant (Smallegange et al., 2011; Xie at. al., 2019). Therefore, the development of an odour blend is required for the surveillance and control of mosquito vectors. Silva et al. (2005) reported that the chemical blend (comprising 480 ml of acetone, 0.96 g of L-lactic acid, and 10 ml of dimethyl disulfide) was effective in attracting *Ae. aegypti* females under controlled laboratory conditions. BioGents sentinel traps baited with a blend of lactic acid, octenol, and isovaleric acid exhibited the greatest percent attraction for *Ae. aegypti* (29.5% ± 14.3%) (Kim et al., 2021). Hexanoic acid attracted more *Ae. aegypti* compared to the commercially available BG-Lure in a study conducted in Kenya (Owino et al., 2015). The combination of binary mixtures, such as lactic acid with acetone or butanone, and mixtures of lactic acid, ammonia, and ketones, has also been reported as an effective lure for female *Ae. aegypti* (Venkatesh et al., 2017). Various synthetic mixtures comprising lactic acid, ammonia, short-chain carboxylic acids, ketones, sulfides, or chloroalkanes have been reported to attract female *Ae. aegypti* (Bernier et al., 2015). The findings emphasize that, *Ae. aegypti* prefers a complex blend over individual components for host-seeking.

These findings have several implications for vector management and integrated pest management (IPM) strategies. The identified chemical blend consisting of acetone, lactic acid, octenol, hexanoic acid, cyclopentanone and ammonium bicarbonate can be incorporated into mosquito traps to enhance their effectiveness in capturing *Ae. aegypti*. Scott-Fiorenzano et al. (2017) showed that incorporation of L-lactic (1%) and 1-octen-3-ol (1%) to a fruit-based sugar bait increased attraction of *Ae. aegypti*. Therefore, the identified synthetic blends might improve the efficacy of attractive toxic sugar baits by enhancing the attractiveness of sugar baits (Kumar et al., 2021). Future research should focus on field validation of these chemical blends under diverse environmental conditions and mosquito population densities. Additionally, exploring the stability and longevity of the formulations in field settings will be critical to ensure their practical utility. Developing cost-effective and scalable production methods for these blends will also be essential to facilitate their integration into vector control programs, particularly in resource-limited settings where mosquito-borne diseases have the greatest impact.

CONCLUSION

The development of a chemical attractant blend for mosquitoes holds promise for improving the effectiveness of vector control tools. By refining the chemical composition and enhancing its applicability in real-world scenarios, this technology can contribute to more effective mosquito population management and disease prevention efforts. The results contribute to the growing body of research supporting the use of olfactory cues to enhance vector management strategies, offering a promising tool for mitigating the global burden of mosquito-borne diseases.

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EFFECTS OF POLYSTYRENE MICROPLASTIC INGESTION ON DEVELOPMENT, ADULT FITNESS, AND REPRODUCTIVE SUCCESS OF *CULEX QUINQUEFASCIATUS* AND *ANOPHELES QUADRIMACULATUS*

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ABSTRACT

Plastic pollution is an escalating global problem that significantly impacts ecosystems. Because plastics are non-degradable, they accumulate throughout the environment as microplastics (MPs). MPs are a major pollutant in aquatic environments, leading to their inevitable ingestion by a wide range of organisms, including mosquitoes. This laboratory study was conducted to determine the effects of MP ingestion by mosquito larvae on the development, adult fitness, and reproductive success of *Culex quinquefasciatus* and *Anopheles quadrimaculatus*. First instar larvae were exposed to different combinations of particle size and concentration of carboxylate-functionalized orange fluorescent polystyrene microspheres; 1 µm and 1000 particles/mL, 1 µm and 100,000 particles/mL, 30 µm and 1000 particles/mL. The results demonstrated that polystyrene MP ingestion impacts larval development, adult fitness (adult size, blood-feeding rate), and reproductive success (fecundity) with differential effects in *Cx. quinquefasciatus* and *An. quadrimaculatus*. The study suggests the possibility of particle size and concentration thresholds that significantly impact the life history traits of different mosquito species, potentially influencing population sizes and vectorial capacities. These observations underscore the need for further investigation into the mechanisms and broader implications of plastic pollution on mosquito populations using environmentally realistic MP concentrations.

Key words: microplastics, *Culex quinquefasciatus*, *Anopheles quadrimaculatus*, particle size, concentration

INTRODUCTION

Plastic is used in almost every aspect of human needs including consumer products, textiles, constructions, electrical and electronics, and machinery. Up to 10% of the plastic produced each year worldwide ends up in the aquatic environment, where it persists and accumulates (Jambeck et al. 2015). Microplastics (MPs), typically less than 5 mm in size, are the end product of plastic waste (Wright et al. 2013, Ziani et al. 2023). Based on the origin, MPs are categorized as primary and secondary MPs. Primary MPs are intentionally manufactured for commercial use such as cosmetics, textiles, and industrial abrasives, and released into the environment in their original size. Secondary MPs resulted in the breakdown of larger plastic items over time due to weathering, sunlight exposure, and other environmental factors (NOAA Marine Debris Program 2009). These MPs can enter the environment through various pathways. They may be released directly into water bodies through wastewater from households and industries. Plastic debris in oceans, rivers, and lakes can break down into smaller particles, contributing to the continuous accumulation of MPs in aquatic ecosystems. Additionally, MPs can be transported by wind (Bullard et al. 2021) leading to their deposition in terrestrial and aquatic environments, and a recent study demonstrated that flying insects can disperse microplastics to new environments (Al-Jaibachi et al. 2018). Microplastic pollution is a growing global environmental concern that has gained attention due to its widespread presence in marine, freshwater, and terrestrial environments (Barnes et al. 2009, Andrady 2011, Browne et al. 2011, Claessens et al. 2011, Rillig 2012, Cauwenberghe et al. 2013, Eriksen et al. 2013, Lusher et al. 2014, Eerkes-Medrano et al. 2015, Huerta-Lwanga et al. 2016) and even in cloudwater (Xu et al. 2024). The most common types of MPs found in the environment include polyethylene, polypropylene, and polystyrene (Ziani et al. 2023).

The consequences of MP pollution are multifaceted and may pose deleterious threats to aquatic organisms worldwide. In aquatic ecosystems, MPs can be ingested by aquatic organisms ranging from plankton to larger animals (Carpenter and Smith 1972, Cole et al. 2013, Cole and Galloway 2015, Lu

et al. 2016, Rist et al. 2016). Exposure of aquatic organisms to microplastics may negatively impact feeding (Wegner et al. 2012, Ogonowski et al. 2016), growth (Au et al. 2015, Jeong et al. 2016), reproductive capabilities (Della Torre et al. 2014, Ogonowski et al. 2016), or survival (Booth et al. 2016, Luís et al. 2015) due to blockage of feeding structures or reduced consumption of prey (Wright et al. 2013, Eerkes-Medrano et al. 2015). Effects such as intestinal blockage, physical damage, histopathological alterations in the intestines, change in behavior, change in lipid metabolism, and transfer to the liver were observed concerning fish (Jovanovic 2017). Thus, the ingestion of MPs can have detrimental effects on the health of those organisms and the food chain, potentially impacting human health (Foley et al. 2018).

Microplastics can be ingested by organisms directly due to confusion with actual food particles or passively during particle filtration for feeding (Collignon et al. 2014). As mosquito larvae are non-selective filter feeders (Merritt et al. 1992), MP pollution may impact their fitness. Studies have shown that mosquito larvae can ingest MPs from their aquatic environment (Al-Jaibachi et al. 2018, Al-Jaibachi et al. 2019). It has been demonstrated that MP contamination is not a limiting factor for oviposition site selection of *Culex pipiens* Linn. (Cuthbert et al. 2019), and in an MP-polluted site, larvae are spontaneously exposed to MP ingestion throughout their development. This ingestion can have physiological and ecological consequences for the larvae. The small size and non-digestible nature of MP particles can lead to blockages in the digestive tracts of the larvae affecting their feeding efficiency, nutrient absorption, and eventually overall development. The presence of MPs can induce stress responses in mosquito larvae (Malafaia et al. 2022). This stress can weaken the larval immune systems and make them more susceptible to diseases or other environmental stressors. MPs can act as carriers for various chemical pollutants (da Costa Araujo and Malafaia 2021). When these MPs are ingested by mosquito larvae, the associated chemicals may be released, potentially causing toxicity (Ribeiro et al. 2019) negatively affecting larval development and survival. A recent study demonstrated the MP ingestion of *Aedes aegypti* Linn. larvae from different concentrations of toothpaste and its adverse effects on larval mortality and adult life history traits (Becker and Xue 2023). Mosquito larvae serve as a crucial food source for many aquatic organisms, including fish and other insects. If mosquito larvae ingest MPs and accumulate associated toxins, it could lead to a transfer of these contaminants up the food chain (da Costa Araujo and Malafaia 2021, Setala et al. 2014), affecting higher trophic levels. As such, there has been a recent surge of interest in mosquitoes regarding the impacts of MP pollution on their life history traits and role as disease vectors.

Studies using *Ae. aegypti* and *Aedes albopictus* Skuse (Simakova et al. 2022, Edwards et al. 2023, McConnel et al. 2024) did not show significant impacts on survivorship and reproduction. *Culex pipiens* (Al-Jaibachi et al. 2018, Al-Jaibachi et al. 2019, Cuthbert et al. 2019), *Culex quinquefasciatus* Say (Li et al. 2024) have been reported on the ontogenic transference of MPs from larvae to adult mosquitoes. The entry of MPs into predators and hosts via mosquitoes, thereby affecting the ecological cycle, has been demonstrated (Cuthbert et al. 2019, Gopinath et al. 2022, Li et al. 2024). Effects on development and growth have also been investigated in the recent literature (Malafaia et al. 2020, Gopinath et al. 2022, Edwards et al. 2023, Griffin et al. 2023, Thormeyer and Tseng 2023). Some studies have reported that MPs cause changes in the mosquito gut microbiome composition, indicating potential implications for their vectorial capacity (Jones et al. 2024, Edwards et al. 2023, Li et al. 2024).

The outcomes of these studies, particularly regarding larval development, varied significantly depending on microplastic (MP) size, concentration, and the developmental stage of the exposed larvae. These variations may also be influenced by the species involved. It is crucial to highlight that research into the specific impacts of MPs on mosquitoes is relatively recent, resulting in a limited understanding of how MP exposure affects traits related to fitness. To contribute to current knowledge, this study investigated the effects of MPs on various life stages of *Cx. quinquefasciatus* and *Anopheles quadrimaculatus* Say under controlled laboratory conditions. *Culex quinquefasciatus* is widely distributed and found in North America, South America, Asia, Africa, the Middle East, Australia and New Zealand (Hill and Connelly 2009) while *An. quadrimaculatus* are primarily seen in eastern North America (Rios and Connelly 2007). They are vectors of agents that cause human diseases, specifically West Nile Virus/lymphatic filariasis and malaria, respectively.

MATERIALS AND METHODS

Mosquitoes. First instar larvae of *Cx. quinquefasciatus* and *An. quadrimaculatus* were insectary-reared (temperature 26± 2°C, relative humidity 80±10% and light: dark 14:10 h) in reverse osmosis water (RO) with larval food (Tetramin® fish food, Tetra GMBH, Germany) and used in experiments conducted at Anastasia Mosquito Control District's Insectary and laboratory in May-June 2023. Larvae received ~1 ml of larval food (12.5% w/v

liquid slurry made using powdered Tetramin flakes) in the first 3 days and ~ 3 ml from the 4th day up to pupation.

MP preparation. Carboxylate-functionalized orange fluorescent polystyrene microspheres (density of 1.05 g cm^{-3}, w/v of 2.5%, excitation/emission wavelengths=304, 530/582 nm) of 1.05 µm (S1) and 30.54 µm (S2) (Lab261, Palo Alto, CA, USA) were utilized in the experiments. All microsphere samples were received as 1% solid suspensions (10 mg/ml) in de-ionized water, containing a trace amount of surfactant and 2 mM sodium azide as an anti-microbial agent. Two concentrations of 1,000 particles/ml (C1) and 100,000 particles/ml (C2) with S1 and one concentration of 1,000 particles/ml with S2 were prepared in reverse osmosis (RO) water. The three MP groups were named C1S1, C2S1, and C1S2.

Test design and procedure. Two experiments were conducted, one with *Cx. quinquefasciatus* and the other with *An. quadrimaculatus*. Each experiment consisted of three MP groups and a control group fed only with Tetramin® fish food (Tetra GMBH, Germany). Each MP group and the control group were run in 7 replicates. Glass pans (Oxo Good Grips, CA, USA) of 750 ml were used throughout the experiment to prevent any external introduction of microplastics into the water. On day 1, 100 first instar larvae were introduced into each glass pan with 500 ml of respective MP solutions. The larvae were exposed to MPs for the total duration of aquatic development but without renewing the MP solutions. Larval food was provided daily as required depending on the larval instar. Five to ten larvae at each instar (2nd to 4th), three pupae, and a few adults from each pan were preserved in 70% methanol for the detection of MPs. The presence or absence of MPs in larvae and adults of *Cx. quinquefasciatus* and *An. quadrimaculatus* was observed using an Olympus FluoView Laser Scanning Biological Microscope (FV1000 IX81 confocal microscope). Fluorescence detection was challenging due to the thickness of the sample and the fact that the MPs were not all in the same z-plane. The presence of MPs in *Cx. quinquefasciatus* larvae were also observed using a DM3500 inverted microscope (Leica Microsystems, Wetzlar, Germany) with a GFP filter and pE-300 ultraviolet light (CoolLED, London, England) and a DFC3000-G attached camera (Leica Microsystems, Wetzlar, Germany). Detection of S1 particles was hindered due to the presence of other autofluorescent particles. Therefore, fluorescence was not always a reliable indicator of the presence or quantity of MPs in the sample. Fluorescence detection in the two species was performed solely to provide evidence of MP ingestion by the larvae used in the study. Based on this fluorescence detection, data analyses were conducted under the assumption that the larvae had ingested MPs indiscriminately.

In the experiment with *Cx. quinquefasciatus*, dead larvae were removed from each pan and the number was recorded once every 2 days. In the *An. quadrimaculatus* experiment, live larvae were counted daily as there were no dead larvae, but a reduction in the number of larvae in pans was observed. Mortality at 1st instar larvae was not determined to prevent the possible mechanical death due to the fragility of the larvae. The first day of pupation in each MP group and the control were noted, and the pupae were counted daily and transferred into adult mosquito cages in 50 ml clear plastic cups.

Pupation rate was determined as the percent pupation of the total number of 4th instar larvae exposed to MPs. Pupae from 7 pans of each MP group were combined into 3 BugDorms of 30x30x30 cm (MegaView Science Co., Ltd., Taiwan) to determine the adult emergence rate in 3 replicates for each treatment. The number of adults that emerged in each cage was recorded and provided with 10% sucrose solution ad libitum. Three days after the emergence of all adults, females were blood-fed with a restrained chicken for 15-20 minutes (following the AMCD Animal Care approved protocol 2005) and the number of engorged mosquitoes was counted. Due to the limited resources and time, females of only one adult cage of each treatment were blood-fed to determine the oviposition rate and fecundity. The total engorged *Cx. quinquefasciatus* were allowed for oviposition in 200 ml white plastic cups with 120 mL RO water, while only 20 *An. quadrimaculatus* were individually placed in mesh-screened large, clear plastic cups (500 mL) with smaller white cups (100 mL) with 20 mL RO water and a Coffee filter (Basket style, 17.5 cm diameter cut into 13 cm diameter) for oviposition. The number of oviposited *Cx. quinquefasciatus* was determined by the number of egg rafts laid over 7 days and 10 egg rafts from each MP group were randomly selected to count the egg numbers. *Anopheles quadrimaculatus* eggs laid on the water surface (over 3 days) were filtered onto the coffee filters and the number of oviposited *An. quadrimaculatus* was determined by the presence/absence of eggs on filter papers. The number of eggs on each filter paper was counted to determine the fecundity. The egg numbers were counted under a stereo microscope (Wofe Digivu TM CVM Stereo NTSC System, Carolina Biological Supply Company, Burtlington, NC). Wing length was used as a proxy of body size (Petersen et al. 2016). The left-wing length of 25 females in each experiment was measured from the alular notch to the longest point of the distal margin under a stereo microscope (Wolfe Digivu TM CVM Stereo NTSC System, Carolina Biological Supply Company, Burlington, NC) using a microscale (1 div=0.1 mm, Minitool Inc., CA, USA).

Data analyses. The data were analyzed using SPSS version 20 (IBM® SPSS statistics® IBM Corporation). Kruskal Wallis test was used to compare the effects of different treatment groups with the control. Pairwise comparisons were conducted using the in-built function of the Kruskal Wallis test to report the significance of differences between any two groups. The significant differences between one data point proportions of MP groups and the control were determined by conducting Crosstab tests. Statistical significance between groups was maintained at $P<0.05$.

RESULTS

First instar larvae of *Cx. quinquefasciatus* and *An. quadrimaculatus* were treated with three groups of different concentration and size combinations of polystyrene MP beads to determine their effects on development, growth, and reproductive success. Figure 1 shows the presence of MPs in some of the larval samples.

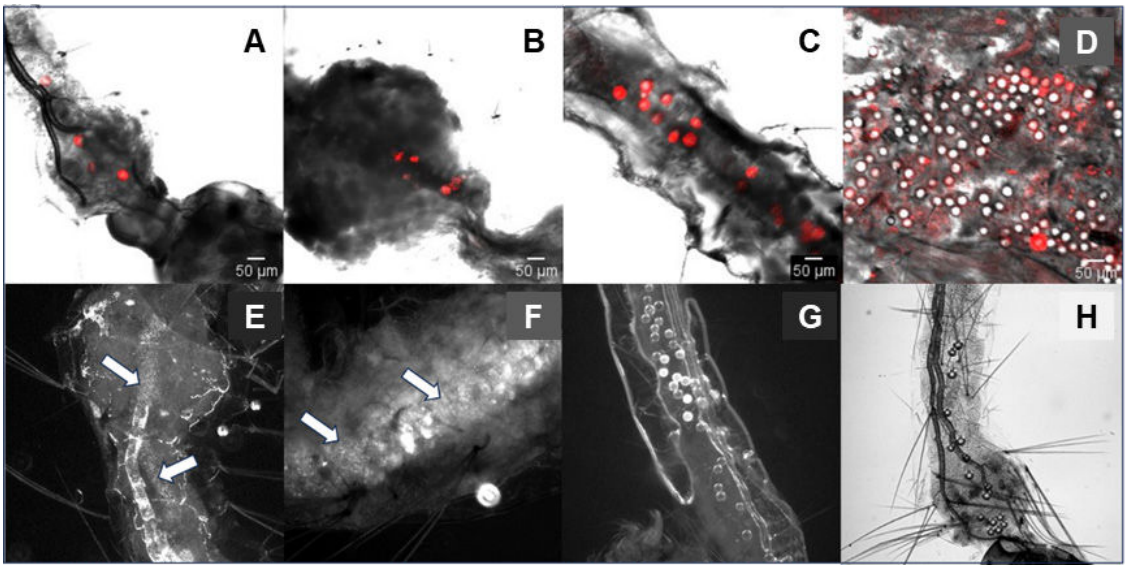

Figure 1. Images of microplastic ingestion of *Culex quinquefasciatus* and *Anopheles quadrimaculatus* larvae exposed to different concentration and size combinations of microplastic particles. (A, B) 2nd and 3rd instar *Cx. quinquefasciatus* respectively with 30 µm diameter particles at 1000 particles/ml. (C, D) 2nd and 4th instar *An. quadrimaculatus* respectively with 30 µm diameter particles at 1000 particles/ml. (E, F) 2nd and 4th instar *Cx. quinquefasciatus* respectively with 1 µm diameter particles at 100,000 particles/ml. (G, H) 2nd and 4th instar *Cx. quinquefasciatus* respectively with 30 µm diameter particles at 1000 particles/ml.

Mortality effects of MP ingestion were determined for the 2nd, 3rd, and 4th instar larvae and pupae of *Cx. quinquefasciatus* and *An. quadrimaculatus*. Significant differences in larval percent mortality at least in one treatment group compared to the control were observed at the 2nd and 3rd instars of *Cx. quinquefasciatus* ($\chi^2_{(3)}=25.25$, $P<0.0005$ and $\chi^2_{(3)}=13.01$, $P=0.005$, respectively) but not at 4th instar ($\chi^2_{(3)}=1.207$, $P=0.751$). Pairwise comparisons confirmed that the mortalities at 2nd (14.7%) and 3rd (14.2%) instars were affected only by C1S1 ($P<0.0005$ and $P=0.039$ respectively) (Table 1). *Anopheles quadrimaculatus* larval mortality was significantly affected at the 3rd instar ($\chi^2_{(3)}=14.25$, $P=0.002$) and only by C1S2 (32.4%) (pairwise comparison: $P=0.009$) (Table 1). Mortality of *An. quadrimaculatus* 2nd instar treated with C2S1 was not determined due to the unavailability of data. Pupal mortality was not affected in either species by any treatment compared to the control ($\chi^2_{(3)}= 2.0$, $P=0.34$ for *Cx. quinquefasciatus* and $\chi^2_{(3)}=1.06$, $P=0.79$ for *An. quadrimaculatus*) (Table 1).

Table 1. Mean larval and pupal percent mortality (determined from the total dead larvae and pupae respectively) of *Culex quinquefasciatus* and *Anopheles quadrimaculatus* treated with different concentration and size combinations of microplastic particles (mean ± standard error) (C1S1: 1µm diameter particles at 1000 particles/ml, C2S1: 1 µm diameter particles at 100,000 particles/ml, C1S2: 30 µm diameter particles at 1000 particles/ml).

	Culex quinquefasciatus				*Anopheles quadrimaculatus*			
	2nd instar	**3rd instar**	**4th instar**	**Pupae**	**2nd instar**	**3rd instar**	**4th instar**	**Pupae**
Control	0	0.7 ± 0.7	0.8 ± 0.8	1.9 ± 0.4	6.0 ± 1.0	13.5 ± 2.5	21.5 ± 4.3	16.6 ± 8.1
C1S1	14.7 ± 2.9	14.2 ± 5.2	0.3 ± 0.3	2.5 ± 0.3	6.5 ± 1.8	13.5 ± 2.1	4.8 ± 3.0	15.1 ± 1.8
C2S1	0	2.5 ± 1.5	0.2 ± 0.2	1.2 ± 0.9	X	16.7 ± 12.5	2.4 ± 1.0	25.1 ± 9.6
C1S2	0	0	0	2.8 ± 1.1	4.4 ± 0.9	32.4 ± 3.7	10.1 ± 5.0	19.1 ± 4.3

x=data not available

Pupation of *Cx. quinquefasciatus* occurred over a period of 6 days. A 6-day pupation rate, calculated as the percent pupation of the total number of 4th instar larvae exposed to MPs was determined for *Cx. quinquefasciatus.* Pupation of *An. quadrimaculatus* also occurred over 6 days. However, since consecutive data were available only for the first 3 days, a 3-day pupation rate was determined for *An. quadrimaculatus.* None of the treatments demonstrated significant effects on the pupation rate of either species ($\chi^2_{(3)}$=5.1, *P*=0.165 for *Cx. quinquefasciatus* and $\chi^2_{(3)}$=4.76, *P*=0.191 for *An. quadrimaculatus*) (Table 2). *Cx. quinquefasciatus* larvae treated with C1S1 achieved 81.0±15.8% of the total pupation on day 2 in contrast to 21.8±6.9% pupation with the control, indicating a significant reduction in development time compared to the control ($\chi^2_{(3)}$=9.701, *P*=0.021 and pairwise comparison: *P*=0.012). Larvae treated with C2S1 and C1S2 achieved 45.6±5.8% and 43.6±7.8% of the total pupation on day 2 respectively without any significant difference from the control pupation. *Anopheles quadrimaculatus* larvae treated with C1S1, C2S1, and C1S2 had achieved, 30.6±8.5, 49.1±9.4, and 35.6±10.0% pupation of the total pupation on day 2 respectively compared to the control pupation of 58.8±8.6%. None of the treatments significantly affected the development time of *An. quadrimaculatus* by day 2 ($\chi^2_{(3)}$=3.23, *P*=0.357). The emergence rate (percent emergence of total pupae exposed to MPs) of the two species was not affected by any of the treatments when compared to the control ($\chi^2_{(3)}$=0.913, *P*=0.822 for *Cx. quinquefasciatus* and $\chi^2_{(3)}$=1.05, *P*=0.0.789 for *An. quadrimaculatus*) (Table 2). A significant reduction in the adult size (in terms of female wing length) was observed in both species in the C1S1 group compared to the control ($\chi^2_{(3)}$=12.753, *P*=0.005 for *Cx. quinquefasciatus* and $\chi^2_{(3)}$=8.748, *P*=0.033 for *An. quadrimaculatus*) (Table 2).

Table 2. Pupation rate, emergence rate, and adult size (wing length) of *Culex quinquefasciatus* and *Anopheles quadrimaculatus* treated with different concentration and size combinations of microplastic particles (mean ± standard error) (C1S1:1µm diameter particles at 1000 particles/ml, C2S1: 1µm diameter particles at 100,000 particles/ml, C1S2: 30 µm diameter particles at 1000 particles/ml).

	Culex quinquefasciatus		*Anopheles quadrimaculatus*	
	Pupation rate	**Emergence rate**	**Pupation rate**	**Emergence rate**
Control	61.8 ± 4.6	95.7 ± 3.0	60.8 ± 10.2	83.4 ± 8.1
C1S1	57.3 ± 7.2	97.2 ± 1.1	34.4 ± 9.8	84.9 ± 1.8
C2S1	69.4 ± 3.4	97.9 ± 0.6	36.7 ± 10.3	74.9 ± 9.6
C1S2	72.5 ± 3.2	98.2 ± 1.4	37.2 ± 8.4	80.9 ± 4.2

There were some notable changes in the blood-feeding rates and oviposition rates of *Cx. quinquefasciatus*. Both blood-feeding rate and oviposition rate significantly increased with C2S1 exposure ($\chi^2_{(1)}$=5.333, P=0.021 and $\chi^2_{(1)}$=11.265, P=0.001 respectively) compared to the control, while there were no significant differences with other two treatments (blood feeding rate-C1S1: $\chi^2_{(1)}$=0.4, P=0.527, C1S2: $\chi^2_{(1)}$=0.148, P=0.700, oviposition rate-C1S1: $\chi^2_{(1)}$=0.147, P=0.701, C1S2: $\chi^2_{(1)}$=0.798, P=0.372). There was no significant difference in the fecundity of *Cx. quinquefasciatus* with any treatment compared to the control ($\chi^2_{(3)}$=1.498, *P*=0.683). Similarly, none of the treatments demonstrated significant differences in either the blood-feeding rate (C1S1: $\chi^2_{(3)}$=0.001, *P*=0.979, C2S1: $\chi^2_{(3)}$=2.272, *P*=0.132, C1S2: $\chi^2_{(3)}$=0.013, *P*=0.909) or the oviposition rate ($\chi^2_{(3)}$=3.243, *P*=0.072 for all three treatments) of *An. quadrimaculatus*. However, the fecundity of *An. quadrimaculatus* treated with C2S1 was significantly lower ($\chi^2_{(3)}$=8.901, *P*=0.031, pairwise comparison: *P*=0.026) (Table 3).

Table 3. Adult fitness traits of *Culex quinquefasciatus* and *Anopheles quadrimaculatus* treated with different concentration and size combinations of microplastic particles (mean ± standard error or percentage) (C1S1:1µm diameter particles at 1,000 particles/ml, C2S1: 1µm diameter particles at 100,000 particles/ml, C1S2: 30 µm diameter particles at 1,000 particles/ml).

	Culex quinquefasciatus				*Anopheles quadrimaculatus*			
	Wing length (mm)	**Blood-feeding rate (%)**	**Ovi position rate (%)**	**Fecundity**	**Wing length (mm)**	**Blood-feeding rate (%)**	**Ovi position rate (%)**	**Fecundity**
Control	3.39 ± 0.02 (n=30)	90.7 (n=75)	66.3 (n=53)	161.2 ± 10.3 (n=10)	3.13 ± 0.06 (n=30)	54.0 (n=189)	100.0 (n=20)	121.5 ± 14.6 (n=20)
C1S1	3.29 ± 0.02 (n=30)	87.3 (n=63)	66.0 (n=47)	163.5 ± 8.0 (n=10)	3.04 ± 0.03 (n=30)	54.1 (n=146)	100.0 (n=20)	118.3 ± 6.5 (n=20)
C2S1	3.34 ± 0.02 (n=30)	98.8 (n=82)	89.5 (n=57)	151.7 ± 7.8 (n=10)	3.06 ± 0.02 (n=29)	62.5 (n=128)	85.0 (n=20)	86.9 ± 10.0 (n=17)
C1S2	3.32 ± 0.03 (n=30)	88.7 (71)	53.3 (n=45)	152.8 ± 9.5 (n=10)	3.08 ± 0.03 (n=30)	53.4 (n=178)	100.0 (n=20)	100.7 ± 6.2 (n=18)

DISCUSSION

Ontogenical transference of MP particles by *Aedes* and *Culex* larvae has been reported in previous studies (Al-Jaibachi et al. 2018, Al-Jaibachi et al. 2019, Cui et al. 2022, Gopinath et al. 2022, Simakova et al. 2022, Edwards et al., 2023, Li et al. 2024). Only a few studies have been published on MP effects on the growth and development of mosquitoes (Al-Jaibachi et al. 2019, Thormeyer and Tseng 2023, Li et al. 2024, McConnel et al. 2024). The present study attempted to contribute to the limited knowledge by reporting new information on *An. quadrimaculatus* and *Cx. quinquefasciatus*. This is the first documentation of MP ingestion in *An. quadrimaculatus*.

Microscopic MP detection indicated the ingestion of both particle sizes and concentrations by *Cx. quinquefasciatus*. Presence of MPs in adult *Cx. quinquefasciatus* corroborates the previous findings on the ontogenic transfer of MPs (Li et al. 2024). With the specific equipment used for *An. quadrimaculatus*, only the large-size MP was detected suggesting that smaller particles could be ingested. The unavailability of evidence for the ontogenic transfer of MP in *An. quadrimaculatus* calls for further investigations.

Most importantly, the study demonstrated the differential effects of the same combinations of particle size and concentration on the two species. The significant mortality of only *Cx. quinquefasciatus* larvae at the small-size, low-concentration MP exposure but not at the same size, high-concentration MP exposure is challenging to explain. This could be attributed to the possible aggregation of smaller MP particles (Wand et al. 2021), which form clumps that affect the ability to ingest by different instars and species. It was estimated that mosquito larval midguts grew 4 to 5- fold between the 1st and 3rd instars (Trager 1937, Ray et al. 2009) suggesting variation in their ability to ingest particles of different sizes. The size of the mouthparts (Bar and Andrew 2013) and obviously the width of the pharynx and esophagus also varies by instar and may affect MP ingestion. If ingested, it could cause larval gut damages (Edwards et al. 2023) or physiological changes (Malafaia et al. 2020) that would result in differential mortality in early instars. The particle size for optimal ingestion by larvae ranges from 0.71 µm up to 1.86 µm for the 1st instar, 7.6 µm for the 2nd and 3rd instars, and 26 µm for the 4th instar, above which sizes ingestion rates declined with increasing size (Dadd 1971). *Anopheles* larvae are generally smaller than *Culex* larvae which indicates a possible difference in particle size selection by different larval instars of the two species. That would explain the differential larval mortality of *Cx. quinquefasciatus* and *An. quadrimaculatus* by the two combinations of the same concentration with small and large particles. The possible size differences in the aggregatory clumps of the two concentrations of the small-size particles might have made a barrier for early instar larvae of *Cx. quinquefasciatus* to ingest a substantial number of particles from the higher concentration. The subsequent effects on adult fitness and reproductive success suggest that particles of both sizes and concentrations are ingested by the late instars (4th and possibly late 3rd) of both species without any mortality effects (both sizes and concentrations were detected in 4th instar large-size particles were detected in the 4th instar *Cx. quinquefasciatus* and the large-size particles were detected in 4th instar *An. quadrimaculatus*). Furthermore, the 3rd instar larvae are known to have a higher feeding rate compared to the 4th instar. This is due to the increased energy requirements as they prepare for the final molt to the 4th instar which reduces their feeding significantly as they approach the pupal stage. In light of all these, it is evident that there should be a threshold particle size and concentration for early larval instars of different mosquito species to be affected by MPs. Once exposed to those thresholds, the physical damage caused by ingesting and accumulating indigestible MPs (Li et al 2024), as well as resulting nutrient deficiencies and related biochemical changes (Malafaia et al. 2020), may cause larval mortality. Griffin et al. (2023) demonstrated 100% mortality of 1st instar *Cx. quinquefasciatus* at 6,000 particles/ml with a range of particle sizes from 1-53 µm. Those that survive an exposure would continue development but likely with fitness costs resulting from certain exposures, especially from high concentrations. The early emergence of *Cx. quinquefasciatus* exposed to small-sized particles at low concentrations in this study may be due to a stress-adaptive behavior that helps them overcome unfavorable conditions caused by the accumulation of MP particles (Malafaia et al. 2020, Edwards et al. 2023). The short wing length (reduced body size) of both species at the same exposure implies poor nutrition during larval development. It corroborates the finding that the adult body weight of *Cx. quinquefasciatus* was reduced by exposure to MPs (Li et al. 2023).

The increased blood-feeding rate of only *Cx. quinquefasciatus* at a specific exposure could be attributed to nutrient deficiencies resulting from that exposure. Although there was no evidence, it could be assumed that the late instar larvae exposed to the higher concentration ingested more MPs (Li et al. 2024). Larvae excrete a large amount of accumulated MPs during pupation (Al-Jaibachi et al. 2019), thus transferring reduced amounts to adults. However, the adults that emerged from larvae exposed to

the higher concentration would still accumulate relatively more MPs. The high accumulation of MPs in those adults may result in low energy metabolism, leading to high feeding rates and corresponding high oviposition rates. In contrast, the blood-feeding rate and the oviposition rate of *An. quadrimaculatus* were not significantly changed by any of the three MP exposures. It could be an indication that any of the three MP exposures did not cause changes in energy metabolism in *An. quadrimaculatus*. Despite the absence of changes in the blood-feeding rate and oviposition rate, a significant change in fecundity was observed only in *An. quadrimaculatus* highlights that there may be more subtle changes (Foley et al. 2018) that selectively impact the reproductive success of this species.

In conclusion, the present study highlights the differential effects of microplastic exposure on *Cx. quinquefasciatus* and *An. quadrimaculatus*. The findings suggest the existence of particle size and concentration thresholds at different life stages that significantly impact life history traits, potentially influencing population densities and vectorial capacities (Jones et al. 2024). Understanding the effects and implications of microplastic pollution is complex and requires further investigation into the mechanisms, thresholds, and implications using environmentally realistic microplastic concentrations.

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REMOA TRI DISPLAYS MINIMAL EFFICACY AGAINST ADULT MOSQUITOES WHEN USED AS AN ACTIVE INGREDIENT FOR TOXIC SUGAR BAITS

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ABSTRACT

Mosquito control programs face many limitations to their control efforts, with one major hurdle being insecticide resistance. A novel adulticide, ReMoa Tri, offers a counter to this limitation, demonstrating efficacy against insecticide-resistant populations of adult mosquitoes. This triple-action adulticide contains two understudied active ingredients (abamectin and C8910). Toxic sugar baits (TSB's) have also proven to display efficacy against resistant populations of mosquitoes and provide a novel method of mosquito control. Here, we examine the efficacy of ReMoa Tri when applied as a TSB product. ReMoa Tri was mixed with a 10% sucrose solution and administered to insecticide-susceptible and resistant strains of *Aedes aegypti* Linn. and *Culex quinquefasciatus* Say at varying concentration levels. The highest mortality belonged to the resistant strain of *Ae. aegypti* with 26.19% at a 20% concentration level. While there were many limitations to this study, dissections confirming consumption of the material and statistical significance between the relationship of dose and mortality indicated limited efficacy of the material when applied as a TSB. Despite these findings, the need for further investigation into the efficacy of abamectin and C8910 is essential to expanding the range of materials available to mosquito control efforts.

Key words: *Culex quinquefasciatus*, *Aedes aegypti*, Toxic Sugar Baits, ReMoa Tri

INTRODUCTION

Integrated mosquito management (IMM) programs are responsible for the control of nuisance and vector mosquitoes within their established territories. Major limitations to the control efforts implemented by IMM programs include insecticide resistance, environmental concerns, and a limited number of control methods (Chaudhry et al. 2019, Rajak et al. 2024). Of these limitations, insecticide resistance is often noted as a priority due to the growing presence of resistance in disease vector species and the reliance of IMM programs on insecticide application as a primary control method (Ranson & Lissenden 2016, Parker et al. 2020, Kondapaneni et al. 2021, Estep et al. 2024, Lopez et al. 2024). As such, the development and application of innovative and novel control measures is often a priority amongst IMM programs and vector control researchers (Obeagu & Obeagu 2024, Rajak et al. 2024, Weng et al. 2024).

One such control strategy which has been gaining interest due to its illustrated efficacy against resistant populations of mosquitoes and potential for limited environmental impact is Toxic Sugar Baits (TSBs)/ Attractive Toxic Sugar Baits (ATSBs) (Stewart et al. 2013, Gu et al., 2020, Diarra et al. 2021, Njoroge et al. 2023). Toxic Sugar Baits exploit the sugar feeding behavior of mosquitoes, which require the carbohydrate as an energy source, by combining a toxin with a sugar source, causing mortality after ingesting the solution (Clements 2000, Foster 1995, Fiorenzano et al. 2017).

An additional new tool within mosquito management is ReMoa Tri ® (Valent Biosciences, Libertyville, Illinois). ReMoa Tri (4% fenpropathrin, 1.5% abamectin, and 1% C8910 Fatty Acid) is a promising new adulticide which has displayed strong efficacy against resistant populations of mosquitoes, likely due to the synergistic properties of C8910 with pyrethroids (Ramadan et al. 2022, Lucas et al. 2024, Unlu et al. 2024). C8910 is a food safe patented mixture of fatty acids which displays repellency towards select dipterans and ticks and has displayed toxicity against mosquito species (Mullens et al. 2009, Dunford et al. 2014, Samuel et al. 2015). Despite the promising and useful applications found within C8910 however, the mode of action of the material is unknown (Reifenrath 2010). Abamectin is an additional important component of ReMoa Tri. Abamectin is isolated from soil dwelling bacteria and is a commonly applied pesticide within agricultural pest control (Feng et al. 2023). Abamectin has

also displayed efficacy as a larvicide and adulticide against mosquitoes within laboratory conditions (Rahman et al. 2024). Furthermore, Clanton et al. (2025) illustrated the efficacy of abamectin as an ATSB against susceptible *Ae. aegypti* in a choice feeding assay.

Given the success of these two novel tools of vector control (ReMoa Tri and TSBs/ATSBs) and the need for continued investigation into TSB/ATSB products and novel active ingredients, it is worthwhile to explore the potential of ReMoa Tri formulated as a TSB product. Anastasia Mosquito Control District (AMCD), the mosquito control authority of St. Johns County FL, has previously illustrated the value of applied TSB products within laboratory and field settings (Xue et al. 2006, Revay 2013, Qualls et al. 2014). AMCD contends with a large population of *Aedes aegypti* L. and *Culex quinquefasciatus* Say within urban habitats of St. Johns County. These species are especially difficult to control given their cryptic oviposition behavior and insecticide resistance status (Dixon et al. 2020, Parker et al. 2020, Estep et al. 2024, Aryaprema et al. 2025). Control difficulties of these two species promotes public health concerns, as *Ae. aegypti* is an important vector of yellow fever, chikungunya, dengue, and Zika, while *Cx. quinquefasciatus* vectors West Nile virus, eastern equine encephalitis, and St. Louis encephalitis (Morris 1988, Souza-Neto et al. 2019, CDC 2024). In this study, we have examined the use of ReMoa Tri formulated as a TSB against susceptible and resistant *Aedes aegypti* and *Culex quinquefasciatus* populations.

MATERIALS AND METHODS

Mosquito Collection and Rearing. Insecticide susceptible *Ae. aegypti* (ORL 1952) and *Cx. quinquefasciatus* (GSV 2002), along with pyrethroid resistant *Ae. aegypti* (Puerto Rico 2012), are maintained at AMCD facilities year-round. To obtain a population of resistant *Cx. quinquefasciatus* (WILD), egg rafts were collected from a field site located in downtown St. Augustine, St. Johns County FL, where a population of the species has been identified as highly resistant to pyrethroids and organophosphates through routine in house resistance monitoring (CDC bottle bioassays and topical assays) (unpublished data). Four-gallon plastic buckets were placed at separate locations (Latitude, Longitude: 29.8838, -81.3144; 29.8829, -81.3133; 29.8818, -81.3132; 29.8829, -81.3124; 29.8822, -81.3115, 29.8813, -81.3105) within a 1.5 km radius. The buckets were then filled with approximately 2 L of hay infused water. Egg rafts were collected 1-2 times per week. Collections occurred during the summer and fall of 2024. All populations (lab and field colonies) were reared within AMCD's climate-controlled insectaries (temperature: 26.6° ± 1° C, RH:70 ± 10%, 14:10 photoperiod (L:D)). Mosquitoes were reared until 5–7-day old adults and were provided a 10% sucrose solution until 24 h before testing, at which point all food and water sources were removed.

Product Formulation. On the same day of testing, ReMoa Tri was mixed with Tween® 20 (Sigma Aldrich, Burlington, Massachusetts) at a 0.1-1% concentration rate to serve as an emulsifying agent. The ReMoa Tri and Tween® 20 solution was then homogenized in a 10% sucrose solution (granular sugar dissolved in RO water). Blue No. 1 Dye (Ingredient Depot, Beauharnois, Quebec, Canada) added at 1% (w/v) provided coloring to allow for the assessment of ingestion of the solution by individual mosquitoes. Tween® 20 and Blue No. 1 Dye were added into the negative control solution of 10% sucrose solution to account for any potential adverse effects attributed by the ingredients. The particular batch of ReMoa Tri was confirmed to be effective against a field colony of *Ae. aegypti* during a semi-field study conducted at AMCD during the fall of 2024 (unpublished data).

ReMoa Tri was tested at 0.1%, 1%, 5%, 10%, and 20% (v/v). The concentration range was chosen following preliminary range finding assessments and was limited at a maximum of 20% due to the material corroding the permeable membrane which restricted contact between the mosquitoes and the material. *Cx. quinquefasciatus* (WILD) were only exposed to 0.1%, 1%, 10%, and 20% concentration levels due to a limited supply of mosquitoes. Concentration levels represent the amount of ReMoa Tri present within the final 100mL solution used in testing.

Table 1. Amount of individual active ingredient (a.i.) per dose of ReMoa Tri (v/v).

Dose	Fenpropathrin (mL)	Abamectin (mL)	C8910 (mL)
0.1%	0.004	0.0015	0.00099
1%	0.04	0.015	0.0099
5%	0.2	0.075	0.0495
10%	0.4	0.15	0.099
20%	0.8	0.3	0.198

Experimental Testing. Ten 5–7-day old adult female mosquitoes were aspirated into 12oz paper cups, affixed with a mesh lid. Each trial consisted of seven technical replicates (7 cups of 10 mosquitoes; 70 mosquitoes per concentration). Hemotek Feeding Membrane ® (Blackburn, United Kingdom) was placed

on the mesh lid as a permeable feeding substrate. This collagen membrane was used in lieu of parafilm, which would dissolve on contact with ReMoa Tri. The Hemotek Feeding Membrane also dissolved at concentrations higher than 20%, which limited the possible range of test concentrations. The respective ReMoa Tri solutions were administered via a saturated cotton ball placed on top of the feeding membrane, with the same cotton ball being used until the completion of the experiment. Mosquitoes were held in a climate-controlled incubator (temperature: 26.6° ± 1° C, RH:70 ± 10%, 14:10 photoperiod (L:D)) for the duration of the testing. Mortality, here defined as the inability to properly stand, maintain normal flight behavior, or respond to stimuli, was observed at 24, 48, and 72 h for *Ae. aegypti* populations and 24 and 48 h for *Cx. quinquefasciatus* populations. The test duration was established by control testing during preliminary range finding, which consisted of mosquitoes of each species and strain being aspirated into holding containers with no sugar source. Mortality of this control was then monitored for 72 h. *Ae. aegypti* (ORL and PR) did not experience any mortality within the period, however, *Cx. quinquefasciatus* (GSV and Wild) experienced >10% mortality after 48 h. Therefore, to ensure mortality was from consumption and not starvation, *Cx. quinquefasciatus* trials were ended at 48 h. Individual mosquitoes were then dissected under microscope to confirm consumption of the material. Consumption was recorded qualitatively, with any amount of blue coloration noted within the thorax or abdomen of the mosquito resulting in a positive recording for consumption rates. Presence of colored fecal droplets was also qualitatively recorded for presence or absence. A total of 3 trials were conducted. Individual trials tested separate cohorts of 5–7-day old mosquitoes with newly formulated solutions. Trials with control mortality above 5% were corrected using Abbots Formula as per W.H.O. guidelines, while trials resulting in control mortality over 10% were repeated (Abbott 1925, W.H.O. 2018).

Statistical Analysis. Pearson correlation tests were conducted in R-Studio (version 4.4.2) to determine statistical significance between the dose of the material and average percent mortality through the Pearson correlation coefficient ($r \approx 1$) and associated p-value ($p<0.05$). To determine the effect of resistant status on mortality, a linear regression analysis was conducted on resistant (WILD & PR) and susceptible (ORL & GSV) strains using the lm() function. A two-sample t-test was then conducted on the estimated slopes and associated standard errors from the linear regression models to determine statistical significance between the two strains ($p<0.05$). To ensure model fit, homogeneity was assessed by plotting the residuals against the predicted values and normality was assessed using the qqnorm() function in R-Studio.

RESULTS

ReMoa Tri formulated as a TSB product displayed limited efficacy against all species and populations. Against all species and strains, at 20% concentration level, the highest observed effect was with *Ae. aegypti* (PR strain), with a 26.19% mortality rate despite 100% consumption levels. Pearson correlation tests conducted on the total percentage for both species and all strains illustrated a statistical significance between the dose of the material and mortality rate ($r=0.608$, $df=21$, $p=0.002$) (Fig 1). At 20% concentration levels, the mean mortality for *Ae. aegypti* ORL and PR were 18.99% and 26.19% respectively. Pearson correlation analysis for ORL and PR revealed that there was no statistical significance between dose and mortality (ORL: $r=0.63$, $df=4$, $p=0.176$; PR: $r=0.804$, $df=4$, $p=0.054$) (Fig 2). However, statistical significance between dose and mortality was confirmed for combined *Ae. aegypti* (PR & ORL) results ($r=0.711$, $df=10$, $p=0.01$ (Fig 3). Results were similar for the *Cx. quinquefasciatus* populations, with 18.35% and 10.95% mortality at 48 h for GSV and WILD populations, respectively. Pearson correlation analysis for *Cx. quinquefasciatus* strains (GSV & WILD) revealed strong statistical significance between dose and mortality for the GSV strain ($r=0.94$, $df=4$, $p=0.005$) and no statistical significance between dose and mortality for the WILD strain ($r=0.417$, $df=3$, $p=0.485$) (Fig 2). Combining *Cx. quinquefasciatus* strains (GSV & WILD) however, did result in an observed statistical significance between dose and mortality ($r=0.723$, $df=6$, $p=0.012$).

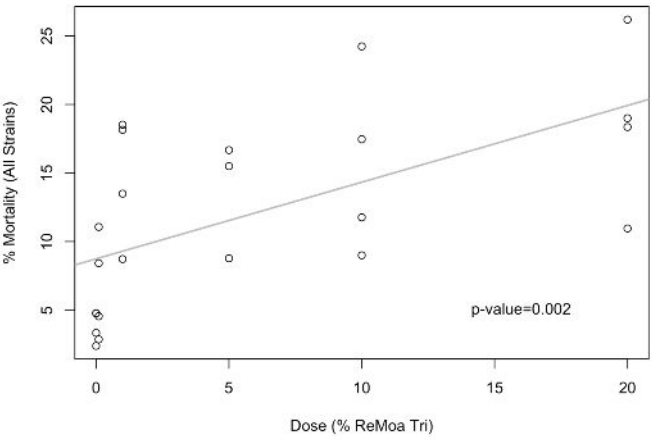

Figure 1. Percent mortality for all species and strains against ReMoa Tri TSB formulation.

All individuals (n=1,260) within both *Ae. aegypti*

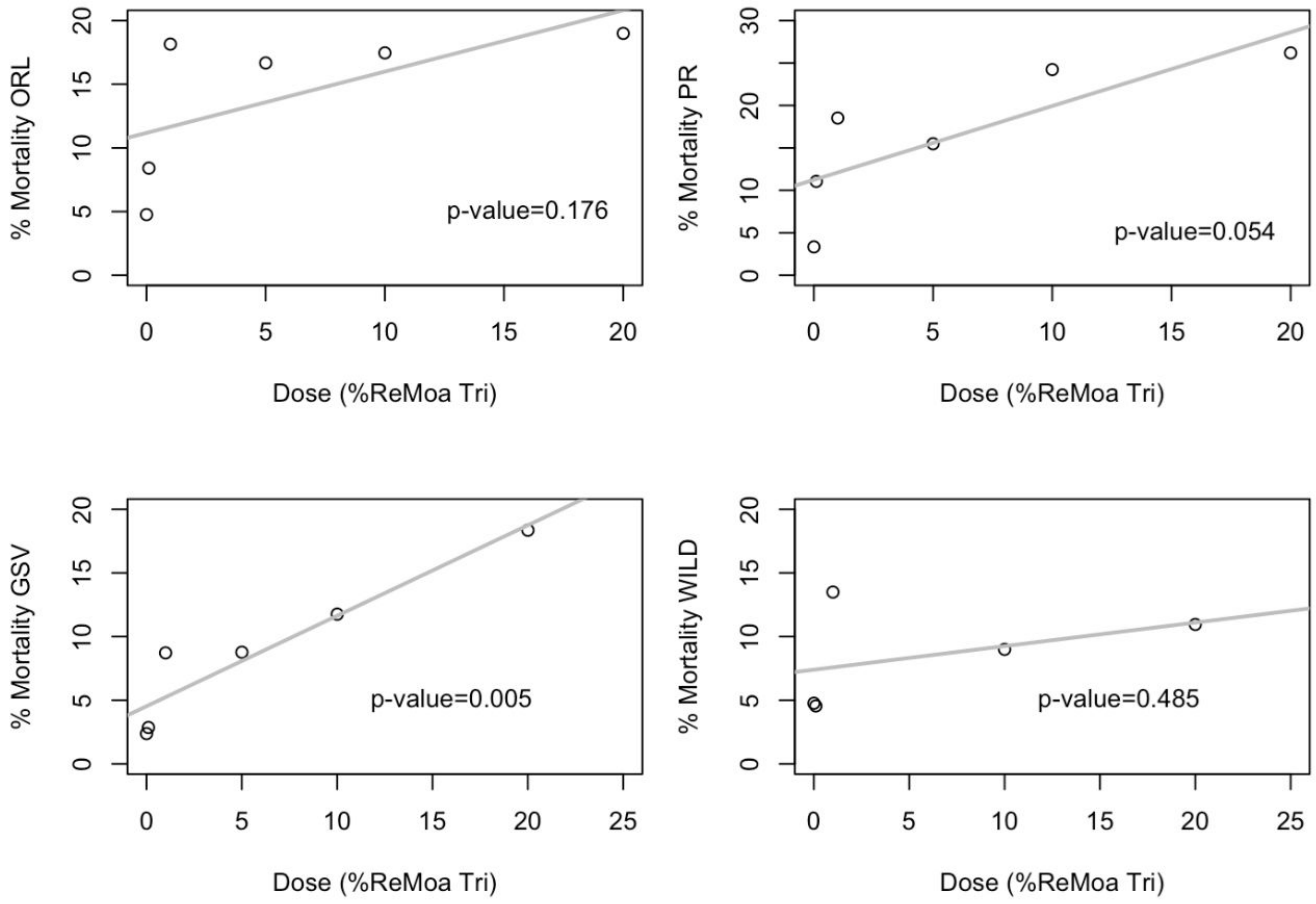

Figure 2. Averaged results for *Aedes aegypti* (ORL & PR) and *Culex quinquefasciatus* (GSV & WILD) against ReMoa Tri TSB product.

strains were confirmed to have ingested the material through dissection. Unlike *Ae. aegypti*, *Cx. quinquefasciatus* (GSV and WILD) did appear to be repelled by the solution as the rate of ingestion of the substance was inversely proportional to the concentration of the solution (Fig 3). This aversion was expressed by the negative control as well however, with 92.38% of the GSV population control consuming the solution and 97.62% of the WILD population control consuming the solution. Colored fecal droplets were present within all treatment and control cups. To investigate the relationship between resistant

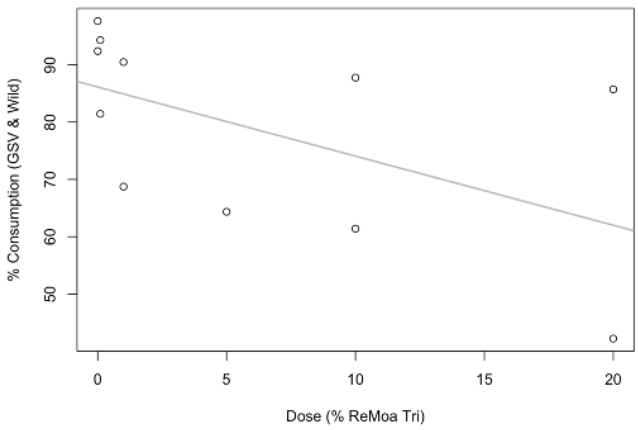

Figure 3. Consumption rates representing the total percent of individuals which consumed material per treatment dose for Cx. quinquefasciatus (WILD & GSV).

status and mortality rate, susceptible (ORL & GSV) and resistant (WILD & PR) strains data were grouped for analysis. Pearson correlation analysis determined statistical significance for the combined susceptible strains (r=0.708, df=10, p=0.01) but did not for the combined resistant strains (r=0.531, df=9, p=0.093). The two-sample t-test assessing the relationship between the linear models of both data sets found the difference between the two groups to not be statistically significant (t-statistic=-0.22, p=0.82).

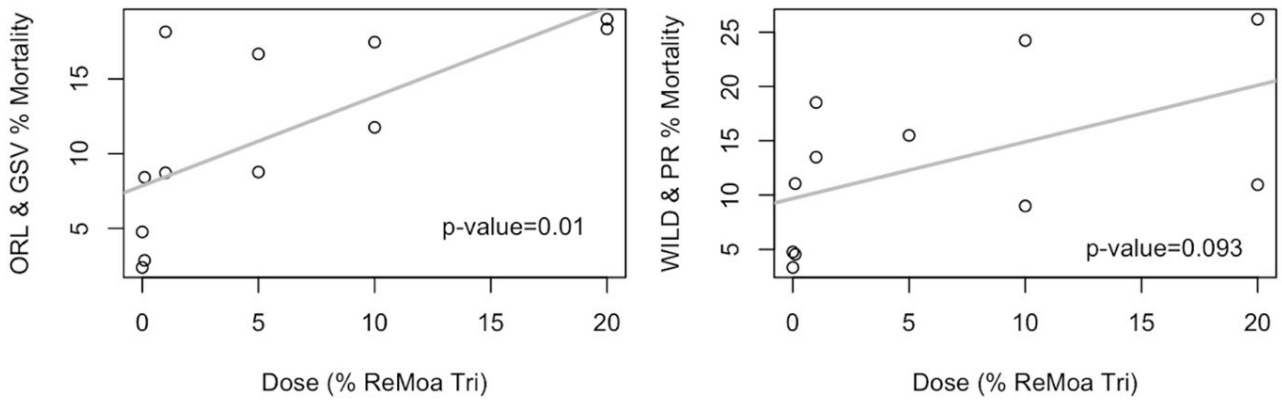

Figure 4. Dose response represented by susceptibility (ORL & GSV) and resistant (WILD & PR).

DISCUSSION

The need for new and effective mosquito control techniques is accentuated as concerns surrounding insecticide resistance and other control limitations grow (Chaudhry et al. 2019, Rajak et al. 2024). Toxic Sugar Baits and Attractive Toxic Sugar Baits provide a complementary control measure to adulticide applications. However, more investigation into materials applied through TSBs/ATSBs is required (Njoroge et al. 2023). ReMoa Tri, a novel adulticide containing understudied active ingredients with unknown modes of action, provides an opportunity to investigate how this formulation of materials may function as a TSB product.

Lucas et al. (2024) determined that ReMoa Tri was highly effective when applied as an ultra-low volume (ULV) adulticide during semi-field trials, achieving 95% and 72-89% mortality at 24 h against pyrethroid resistant *Cx. quinquefasciatus* and *Ae. aegypti*, respectively. Unlu et al. (2024) similarly found ReMoa Tri to display efficacy against resistant *Cx. quinquefasciatus*, with a 65.1 ± 7.2% mortality occurring at 24 h and 85.3 ± 9.1% occurring at 48 h. When administered as a TSB against susceptible and resistant populations of *Ae. aegypti* and *Cx. quinquefasciatus* the highest mortality rate achieved was 26.19%.

The explanation behind the limited efficacy is difficult to deduce, however, some assumptions can be made given the mechanism of action of the material and the results. ReMoa Tri's proposed mechanism of action, provided by Valent Biosciences, begins with the adherence of C8910 to the cuticle of the mosquito. The abamectin and fenpropathrin can then bind to the C8910, allowing for the absorption of the material through the cuticle. Once absorbed, abamectin works by interfering with the glutamate-gated chloride channels, while fenpropathrin works as a pyrethroid, disrupting the voltage gated sodium channels. For ingestion of sugar meals by female mosquitoes, the labellar response begins after the sugar-sensitive sensilla are stimulated, resulting in the labella beginning the process of consumption, storing the sugar solution within the crop (Clements 2000). Within the crop, the sugar source will begin to be quickly hydrolysed by salivary enzymes (Clements 2000). These digestive processes, along with further digestive actions within the midgut, may have disrupted the mechanism of action, which ReMoa Tri attributes its success to. However, Clanton et al. (2025) found abamectin to be effective during ATSB choice assays, resulting in over 90% mortality after 24 h. Additionally, other investigations have found other commonly used adulticide active ingredients to be effective

when applied as a TSB/ATSB (Allan 2011, Shin et al. 2011, Fiorenzano et al. 2017). Abamectin has initiated avoidance behavior in some insects, which may have resulted in sub-lethal exposure (*Ae. aegypti* strains) or avoidance (*Cx. quinquefasciatus* strains) within this experiment (Du et al. 2023). However, confirmation of consumption through dissections and an indication of digestion through the presence of fecal deposition indicates a lack of avoidance, especially for the *Ae. aegypti* strains.

While the inability to expand beyond the 20% dose rate provides a limitation to this study and its analysis, mortality would be expected to be higher, especially given the 100% consumption rate for all *Ae. aegypti* (ORL & PR) tested. Furthermore, the analysis between all species and strains against the dose indicated statistical significance ($p=0.002$), illustrating correlation between the variables, providing further evidence for our determination of the limited efficacy of this material when applied as a TSB. When examining the significance of the correlation between dose and percent mortality per strain, the results are inconsistent, likely due to the ineffectiveness of the material applied as a TSB, increases in p-value due to a decrease in the sample size ($df< 5$), or both. Finally, the two-sampled t-tests assessing the significance of the linear models for the susceptible populations (ORL & GSV) and the resistant populations (PR & WILD) did not indicate any significance, illustrating no significant difference of resistance status on the efficacy of the TSB. With these data we can infer that ReMoa Tri has limited efficacy when formulated as a TSB product.

As mentioned above, limitations to this study begin with the inability to exceed 20% of formulated product for the testing dose. Due to this reason, we were unable to determine the lethal concentration required for 50 or 90 percent mortality (LC_{50} and LC_{90}). An additional limitation to this study was the unknown consequences of mixing the product with sugar water and tween, as per label instructions the material is only to be mixed or diluted with a proprietary product offered by Valent Biosciences. Therefore, the formulation of the material itself during testing may have been affected. Finally, *Cx. quinquefasciatus* did not readily consume the solution. A negative correlation was observed between dose level and average consumption with 81.42% of GSV individuals exposed to 0.1% consuming the material, but only 42.22% of the individuals consumed the highest dose level of 20%. A similar, but less drastic trend was seen with the WILD population, with 94.29% consuming on the 0.1% solution and 85.71% consuming the 20% solution. Therefore, a repellency factor is implied against *Cx. quinquefasciatus*. As such, the mortality experienced by the *Cx. quinquefasciatus* populations, especially the SUS population, is not truly representative of a dose-response

Despite the results found here, the investigation into C8910 and abamectin as potential active ingredients used within mosquito control applications, including TSB/ATSB formulation, needs to be conducted. Both these materials provide novel tools in mosquito control and have illustrated efficacy in previous investigations (Samuel et al. 2015, Rahman et al. 2024, Clanton et al., 2025). These valuable tools can provide mosquito control operations with new novel control efforts, resulting in more efficient and thorough treatment of disease vectors and nuisance mosquitoes.

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TOXICITY OF A NOVEL FORMULATION OF BIGSHOT MAXIM AGAINST *AEDES AEGYPTI*

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ABSTRACT

A series of laboratory evaluations were conducted to assess the ADULTICIDAL efficacy of a non-commercial, experimental formulation of BIGSHOT Maxim (PreVasive USA, LLC) against *Aedes aegypti* Linn. mosquitoes. The formulation is a glycerine-based polymer microencapsulated cedar, thyme, cinnamon, peppermint, and lemongrass organic oils (18.8% active ingredient by weight). Efficacy of the microencapsulated formulation was evaluated via CDC bottle bioassay and wind tunnel testing and compared against the original BIGSHOT Maxim formulation. Three bottle bioassay trials were conducted evaluating concentrations of both test materials at 13.3, 6.6, 4.4, and 3.3 µg/cm^2 of active ingredient (A.I.), respectively. Four premade solutions were provided for the wind tunnel testing. These were prepared by mixing 4.0, 7.39, 14.1, and 29.57 mL of an 80 g/L of pyrethrum and 480 g/L of PBO solution into the novel BIGSHOT Maxim formulation to make 100 mL total volume. Bottle bioassay results for the microencapsulated formula ranged between 27.3-93.0% yielding 1h mortality greater than 80% at concentrations above 6.6 µg/cm^2 demonstrating strong efficacy but failed to exceed the original formulation. Overall wind tunnel mortality at 24h was low, ranging between 10.0-22.0%.

Key words: adulticide, nanoformulation, microencapsulation, essential oil, cedarwood oil

INTRODUCTION

Mosquito-borne diseases have historically been a major concern in Florida and will continue to be of public health importance as changes in climate, urbanization and globalization are predicted to drive up rates of disease transmission (Patterson 2016). However, sustained use and overreliance on pyrethroids and organophosphates for adult mosquito control has led to the widespread development of insecticide resistance in several species across the state (Lucas et al. 2020, Mundis et al. 2020). Botanical insecticides offer a potential solution to combat resistant development. Also, these insecticides pose minimal environmental impact and are exempt from FIFRA regulatory approval (40 C.F.R §152.25, 2015). Many botanical ingredients, such as essential oils and/or their constituent chemicals, have demonstrated promising bioactivity such as repellency (Choochote et al. 2007), toxicity (Sarma et al. 2019), and synergism (Tong and Bloomquist 2013) when screened under laboratory conditions against adult mosquitoes.

BIGSHOT Maxim (PreVasive USA, LLC, Oakwood, GA) is a botanical insecticide marketed for mosquito and tick control, which uses cedar oil as the primary active ingredient (AI). Cedar oil has demonstrated adulticidal, larvicidal and repellent effects against different public health arthropods (Cetin et al. 2009, Ramar et al. 2014, Khanna and Chakraborty 2018). It also has precedence as a mosquito control product having previously been explored for use as a spatial spray insecticide under semi-field conditions demonstrating 100% efficacy against *Aedes aegypti* in an ultra-low volume treatment at a concentration of 70 ml/liter and an application rate of 8 ml/ha (Bibbs et al. 2019).

Nonetheless, the commercial production of mosquito control products using botanical essential oils has remained limited, highlighting a gap between laboratory efficacy and commercial feasibility. This can partially be attributed to economic and practical challenges, as essential oils can be difficult to source reliably and cost-effectively (Isman 2006). However, essential oils are also inherently volatile and susceptible to external conditions such as light, temperature, and humidity, which limit their efficacy under field conditions

(Turek and Stintzing 2012). To overcome these limitations, essential oils are commonly microencapsulated during formulation to enhance their delivery, stability, and effectiveness (Sousa et al. 2022).

Microencapsulation can broadly be defined as the packaging of a chemical at the micron scale within a secondary matrix layer. Decreasing particle size greatly increases the surface area to volume ratio, which can improve delivery of insecticides by increasing water solubility, dispersal uniformity, and dissolution rate (Alonzo et al. 2014, Zhang et al. 2019). Furthermore, highly volatile essential oils are prone to evaporation, oxidation, and photodegradation which can reduce their potency and effectiveness. Composition of the protective matrix layer can be selectively formulated to minimize degradation loss, thereby extending treatment duration and enhancing the likelihood of AI reaching its target. Microencapsulation is therefore complementary to essential oils and work to minimize their weaknesses as insecticides.

Previous work evaluating BIGSHOT Maxim against different mosquito and tick species have demonstrated its effectiveness as a pesticide (Bangonan et al. 2022, Bangonan et al 2023). BIGSHOT Maxim also demonstrates moderate efficacy against a pyrethroid resistant strain of *Ae. aegypti*, indicating it may be a useful tool in resistance management strategies (Rodriguez et al. 2022). Building on previous work, the purpose of this study was to investigate the efficacy of a microencapsulated formulation of the BIGSHOT Maxim against *Ae. aegypti* mosquitoes. Additionally, the study examines the efficacy of BIGSHOT Maxim applied in combination with pyrethrum and PBO.

MATERIALS AND METHODS

Mosquitoes. Laboratory studies were conducted using colonized lab-reared *Aedes aegypti* (Linn.) mosquitoes (ORL1952 strain) obtained from the United States Department of Agriculture (USDA), Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, FL. The mosquitoes were reared at Anastasia Mosquito Control District (AMCD) insectaries maintained at 80 ± 2 °F, 80 ±10% relative humidity, and a 14L: 10D photoperiod. Adult mosquitoes were provided 10% sucrose solution *ad libitum* and blood fed using a restrained live chicken to procure eggs. Larvae were reared in plastic trays (22 x 17 x 3") on a diet of Tetramin Tropical Flakes (fish food) powdered and administered in a 1:5 food-to-water slurry. Non-blooded female mosquitoes (5-10 days) were used in the study.

Test item. BIGSHOT Maxim is a commercially available botanical insecticide marketed for mosquito, tick, and agricultural pest control. This is a 25(b) EPA-exempted product, which at the time of testing contained the following AI: cedar oil (15.2%), thyme oil (1.57%), cinnamon oil (1.57%), peppermint oil (0.23%) and lemongrass oil (0.23%). Two formulations of BIGSHOT Maxim were provided for testing: (1) the original product, (2) a novel, non-commercial glycerine-based polymer encapsulated formulation. The microcapsules measured approximately 7-11 µm in diameter.

Bottle Bioassay. The efficacy of a microencapsulated formulation and commercially available version of BIGSHOT Maxim were compared using CDC bottle bioassays against *Ae. aegypti* mosquitoes (Brogdon and McAllister 1998). The standard methodology was modified to include five replicates per concentration (1:50, 1:100, 1:150 & 1:200) of both BIGSHOT formulations. Ten to twenty mosquitoes per replicate were aspirated into each bottle. Test concentrations of the respective formulations were prepared via serial dilution into acetone. Three trials were conducted using the microencapsulated BIGSHOT Maxim, while two trials were performed with the original formulation. The interior surface of 250mL glass Wheaton bottles were evenly coated with 1 mL of the four test concentrations for a treatment of 13.3, 6.6, 4.4, and 3.3 µg/cm^2 of AI, respectively and air dried for one hour. A negative control with 1 mL of acetone and positive control of permethrin (CDC diagnostic dose) was also included. Mortality was observed at 1h and 24h post-treatment.

Wind Tunnel Bioassay. A modular wind tunnel described by Bibbs et al. (2020) was configured to conduct a spatial spray bioassay against adult female *Ae. aegypti* to assess the efficacy of four different experimental formulations of pyrethrum, PBO and the microencapsulated BIGSHOT Maxim formulation, which were provided to AMCD for testing. These were prepared by mixing 4.0, 7.39, 14.1, and 29.57 mL of an 80 g/L of pyrethrum and 480 g/L of PBO solution into the novel BIGSHOT Maxim formulation to make a 100 mL total volume. Final concentrations of the four respective solutions were: (1) 14.6% cedar oil, 0.32% pyrethrum, and 1.92% PBO; (2) 14.1% cedar oil, 0.59% pyrethrum, and 3.55% PBO; (3) 13.1% cedar oil, 1.13% pyrethrum, and 6.77% PBO; and (4) 10.7% cedar oil, 2.37% pyrethrum, and 14.19% PBO.

Three trials were conducted during this experiment. For the bioassay, around fifteen female *Ae. aegypti* were aspirated into cylindrical paper cages measuring 10 cm in diameter. Cages were inserted into the wind tunnel 1.2 m downwind from the point of application. The

cross-sectional area of the wind tunnel is approximately 0.25 m^2. 100 µL of each test concentration was atomized into the wind tunnel using a Terminator Air-Shear nozzle (ADAPCO, Sanford, FL) supplied with 100 psi of compressed air. This volume was selected to evaluate PBO application rates of 1, 2, 4 and 8 oz/acre. Constant airflow of 0.3 m/s towards the mosquito cages was pulled through the wind tunnel by a blower assembly (DC OEM Specialty Blower 3HMH7, W. W. Grainger, Inc., Lake Forest, IL) and exhausted outside the building. Cages were removed 60 seconds after treatment and placed into an incubator maintained at 80 ± 2 °F, 80 ±10% relative humidity, and a 12L: 12D photoperiod. Treated mosquitoes were provided 10% sucrose solution via saturated cotton balls. A negative control of water and positive control of BIGSHOT Maxim (neat) treatment were also included. Mortality was observed at 24h and 48h post-treatment.

Data Analysis. Data was corrected for control mortality above 5% using Abbott's formula (Abbott 1925). Differences in mean mortality between the original and microencapsulated formulations were analyzed in R (version 4.3.2) using the Welch's two sample t-test.

RESULTS

For the bottle bioassay, a total of 15 replicates per concentration per formulation across three trials using the microencapsulated BIGSHOT Maxim and two trials with the original formulation. The average number of mosquitoes per bottle for the microencapsulated formulation (n=1,141) was 15.2 whereas the original BIGSHOT Maxim (n=789) was 15.8. Mortality for the microencapsulated BIGSHOT Maxim at 1h post-treatment ranged between 20.4-86.2.0% while 24h results exceeded 95% for all concentrations against *Ae. aegypti* (Table 1). In comparison, the original BIGSHOT Maxim formulation yielded higher mortality across all four equivalent concentrations ranging between 78.4 – 100% at 1h post-exposure. At 24h post-exposure, BIGSHOT Maxim demonstrated 100% mortality at all four concentrations. Bottle bioassay treatments using the original formulation yielded significantly higher mortality across all concentrations except for 3,760 µg per bottle when compared to the microencapsulated formula.

Overall wind tunnel mortality was low. At 24h post-treatment mortality ranged between 10.5-22.0% for *Ae. aegypti.* At 48h post-treatment, mortality increased slightly to 19.6-28.0% (Fig. 1). A higher dosage is required to determine the lethal concentration of the formulation.

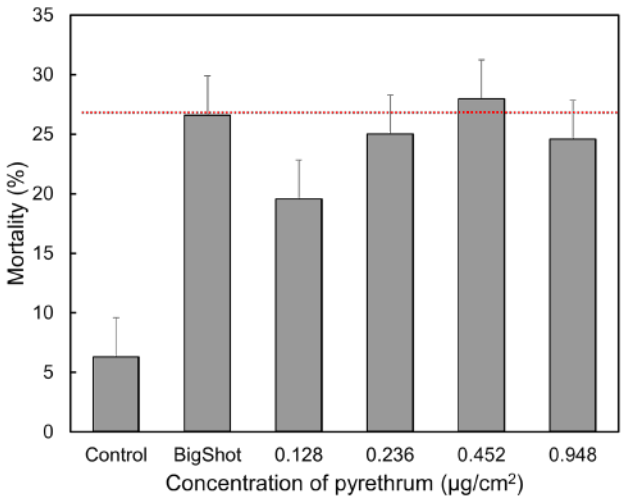

Figure 1. Percent mortality (±SE) at 48h of adult *Aedes aegypti* treated with different concentrations of pyrethrum, PBO and microencapsulated BIGSHOT Maxim in wind tunnel bioassay. Negative control of water and original BIGSHOT Maxim formulation (neat) were included.

Table 1. Percent mortality (±SE) at 1h of adult female *Aedes aegypti* treated with two different formulations of BigShot Maxim and an acetone control using the CDC bottle bioassay assay (n= total number of mosquitoes tested). Significance between the two formulations across each concentration were compared using Welch's two sample t-test.

		Microencapsulated BIGSHOT Maxim		Original BIGSHOT Maxim		
Total	Conc. (µg/ bottle)	n	Mean percent mortality at 1h	n	Mean percent mortality at 1h	Mean Difference Statistic (t-test)
Control	0	232	6.9±6.7%	168	0%	
1:200	940	214	20.7±6.5%	139	78.4±4.1%	t = -6.31, p < 0.001
1:150	1250	205	20.4±3.6%	148	90.5±2.3%	t = -14.85, p < 0.001
1:100	1880	247	74.9±5.1%	166	100%	t = -29.39, p = 0.003
1:50	3760	243	86.2±4.6%	168	100%	t = -1.39, p = 0.19

DISCUSSION

Aedes aegypti bottle bioassay exposure to both formulations of BIGSHOT Maxim resulted in high levels of mortality. However, the microencapsulated BIGSHOT Maxim did not outperform the original formulation as expected. The bottle bioassay methodology was selected for use because BIGSHOT Maxim is marketed as a barrier spray product. Although the testing was primarily focused on the 1h efficacy of both products, mortality observations were extended to 24h. Test mosquitoes, however, were not transferred into clean holding cages and thus were exposed continuously for the duration of the test period. The original intent of the sponsor was to formulate a nanoencapsulated product to further enhance reactivity, but characterization of the capsules revealed they were only in the micrometer range. Though this was merely a preliminary study to see if the original BIGSHOT Maxim could be improved via encapsulation, mortality was not enhanced. Despite this, the novel formulation still produced high levels of mortality at 1h of exposure and could still be worth pursuing as microencapsulation can extend the residual activity of essential oils by preventing photodegradation and premature volatilization (Sousa et al. 2022). In this regard, a more practical aspect of this insecticide to examine would be stability and longevity, examining repeated *Ae. aegypti* exposure and mortality to the same treated surface over several weeks.

In contrast, the wind tunnel bioassay produced low levels of mortality overall and did not exceed 28% at 48h post-exposure for *Ae. aegypti*. Increasing concentrations of pyrethrum and PBO into the test solutions did not yield increased mortality. Furthermore, additions of pyrethrum and PBO did not noticeably improve efficacy above the baseline BIGSHOT Maxim when applied on its own. As the test material used in the wind tunnel bioassay were prepared specifically for the study, we were unable to evaluate additional concentration mixtures to establish the dose-response curve of BIGSHOT Maxim as a spatial spray. It would also have been beneficial to include pyrethrum and PBO alone as a positive control. However, this work was conducted as preliminary study to evaluate the potential of BIGSHOT Maxim as a potential spatial spray insecticide.

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TOXIC CONTACT TARGETS (TCTS) FOR CONTROLLING SAND FLIES AND REDUCING *LEISHMANIA* IN AN OASIS ECOSYSTEM

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ABSTRACT

This study aimed to assess the effectiveness of Toxic Cloth Target (TCT) units in controlling blood-seeking sand flies (*Phlebotomus papatasi*) and reducing infection rates of *Leishmania* in two isolated oases in the lower Jordan Valley, Israel. The treated oasis deployed TCT units from May to November 2011, while the untreated control oasis received no intervention. Sand fly populations were monitored using non-baited CDC traps and infection rates were determined by dissection. In 2012, no treatments were applied, but monitoring continued to observe population trends.

Within 2 weeks of deploying TCTs, sand fly populations at the treated oasis decreased significantly, while populations at the control oasis slightly increased. Over the remainder of 2011, treated oasis populations continued to decline despite reductions in TCT numbers and monitoring frequency, while control oasis populations rose above pre-treatment levels. Infection rates, initially 11.4% at the treated oasis and 6.6% at the control, dropped by 88.3% at the treated oasis after TCT application but increased by 21.7% at the control. During the next 5 months, only 1 of 764 sand flies from the treated oasis was infected, whereas the control infection rate was 7.7% . In 2012, treated oasis populations remained lower than those in the control, except during the final months of monitoring when control populations declined slightly below treated levels.

The findings demonstrated that TCT units effectively reduced sand fly populations and infection rates in isolated conditions. Further research is needed to evaluate their potential for broader applications in areas with widespread sand fly populations.

Key words: *Phlebotomus papatasi*, control, CO2, pesticides, traps, Israel

INTRODUCTION

Phlebotomine sand flies are best known for transmitting *Leishmania*, but they are also vectors of phleboviruses and some bacteria like *Bartonella* (Comer and Tesh 1991, Ashford 2001, Birtles 2001). Leishmaniasis is endemic in 88 countries (22 in the Americas) with an estimated annual incidence of 1-1.5 million cutaneous and 0.5 million visceral cases (Desjeux, 2001). Increasing risk factors such as climate change and broadening human habitation in relation to sand fly habitats are making leishmaniasis a growing public health concern and an emerging disease in urban environments (Oumeish, 1999; Ashford, 2000; Vivero-Gomez *et al.*, 2024). For most *Leishmania* species, humans are a dead-end host, and accordingly, medical cure of patients does not affect transmission (WHO, 1979). At the same time treatment or elimination of dogs, the main domestic reservoir of zoonotic visceral leishmaniasis, or certain rodents, the main reservoir of cutaneous leishmaniasis, is also not achievable (Lewis and Ward, 1987). In the absence of vaccines, it appears that vector control is the only practical approach to interrupt the cycle of transmission (Alexander and Maroli, 2003).

Sand flies are biological vectors of *Leishmania* and the parasites must metamorphose inside the fly before the fly can become infective (Killick-Kendrick 1999). Female sand flies typically begin to blood feed 48 h after eclosion, and under optimal laboratory conditions, at least four days are required for an infection to mature for parasites to be transmitted by the fly (Dye *et al.*, 1987). *Phlebotomus papatasi* is known to blood feed at any stage of follicular development, and repeated feeding on blood during a single gonotrophic cycle is common under natural conditions (Magnarelli *et al.*, 1984; Schmidt and Schmidt, 1965; Denlinger *et al.*, 2016). Compared to mosquitoes, sand flies have a long, temperature-dependent developmental cycle; even under favorable conditions (Chelbi and Zhioua, 2007). They lay fewer eggs in a lifespan than mosquitoes, and it is commonly accepted that most sand fly species only move relatively short distances (Killick-Kendrick 1999; Orshan et al., 2016). Sand flies typically develop in the ground, which makes it difficult to locate and almost impossible to control the immature stages (Feliciangeli, 2004). Adult sand flies exhibit a distinctive hopping behavior rather than continuous flight when moving from their burrows to a potential host. Unlike mosquitoes, which rely on sustained flight to locate and approach their hosts, sand flies tend to make short, erratic jumps, often staying close to the ground. This behavior is influenced by their small size, weak flight muscles, and preference for sheltered environments, such as cracks, burrows, and leaf litter (Halada *et al.*, 2018; Kumari *et al*, 2025). Because sand flies rely on hopping and crawling, control strategies differ from those used for mosquitoes. This behavior makes sand flies vulnerable to any measures that constantly reduce a population (Schlein and Müller 2010).

Common measures to control adults include insecticidal barrier treatments, residual spraying, insecticide treated nets, application of repellents/ insecticides to skin or to fabrics, and insecticide impregnated dog collars (Perich *et al.*, 1995; Vieira and Coelho 1998; Kroeger *et al.*, 2002; Courtenay *et al.*, 2007). Although effective in urban areas with high concentrations of sand flies, the efficacy of surface residuals depends on the exposure of sand flies to a toxic dose of insecticide before the opportunity to bite occurs. Other possible reasons for the ineffectiveness of sprayed insecticide are repellent effects, resulting in insufficient contact time for exposure to a lethal dose or lack of sufficient insecticide in a thin superficial outer layer on the sprayed surface (Alexander and Maroli 2003).

This study evaluated the effectiveness of Toxic Cloth Target (TCT) units in reducing sand fly populations and *Leishmania* infection rates in two isolated oases in the Jordan Valley.

MATERIAL AND METHODS

Study sites. The study was conducted in Israel, north of the Dead Sea, in the lower Jordan Valley 200 m below sea level. This region belongs to the Saharo-Arabian phyto-geographical zone, an extreme desert, with an annual precipitation of 50 to 100 mm restricted to winter, and average temperatures averaging around 20°C from the end of September through early April, to > 30°C from May through August (Ashbel, 1951; Beaumont *et al.*, 1976; Danin, 1988). We selected two similar, uninhabited oases, known for their high sand fly populations. Both sites, covering about 20 hectares, are neglected date plantations (*Phoenix dactilifera*,) with thick natural undergrowth. In these oases, large populations of *P. papatasi* are found in the colonies of sand rats, *Psammomys obesus.* Results from previous studies in the area documented the predominance of *P. papatasi* (Schlein *et al.*, 1984; Müller and Schlein, 2004). Research conducted over the past few decades has consistently shown that infection rates of *Leishmania major* in local sand fly populations in the Lower Jordan Valley are typically around 10% but can be as high as 56% (Schlein *et al.*, 1982). Additionally, *Psammomys obesus*, the primary reservoir host, exhibited an infection rate as high as 93%

(Schlein *et al.*, 1982). This prevalence in the Lower Jordan Valley is considered a common occurrence rather than an unusually high rate (Yuval, 1991).

When the study was performed, the annual winter vegetation had already been grazed, and most of the remaining vegetation consisted of small bushes mainly *Prosopis farcta*, *Atriplex halimus*, *Tamarix nilotica*, and *Suaeda asphaltica* growing among the date trees. There are no open water sources at either oasis, therefore animals like gazelles, porcupines, and hares are scarce. Local Bedouins with their livestock avoid the sites at night because of the high sand fly biting pressure. The two oases were 7 km apart and separated from other suitable sand fly habitats by at least 2 km of hyper-arid, almost barren desert.

Toxic Contact Targets. The TCT used in this study was a modification of the Blue Rhino SkeeterVac SV3100 (Blue Rhino Premier LLC, Winston-Salem, NC, USA) developed as a consumer product for mosquito control (Kline and Lemire, 1998). The current study evaluated Insecticide-Impregnated Shade Cloth Targets, which were treated with lambda-cyhalothrin for the control of sand flies.

TCTs consist of a combustion unit with a filter system allowing them to be operated with propane or butane, or mixtures of the two. In our study, the TCT units were operated with propane in 17.8-L tanks that were exchanged every 3 weeks, well before all of the gas was consumed. The combustion unit, which supplies 500 cc of CO_2/min, is fixed on a stand with a frame that also supports a cylindrical, closed-top, disposable, black textile target. The target is 50 cm in diameter x 90 cm high with the bottom edge 20 cm above the ground. This allows flies to land on the outer target surface or enter it from below (Fig. 1). The target is made of a UV-stabilized combination of woven plastic, fabric that was impregnated with deltamethrin. The fabric was treated with an EC formulation (120 gm/1) of deltamethrin at 0.2 g A.I./m^2. TCT's were fabricated by attaching the insecticide impregnated cloth to upper and lower 5 cm wide bands of tubing using metal screws. The targets were constructed to allow for a 10 cm overlap of cloth, which could be removed for bioassay purposes. The upper surface of the cylinder was also covered with insecticide impregnated shade cloth. The bottom was left open to allow sand flies to enter and rest on the inner surface of the target. The amount of insecticide mixture required to treat the shade cloth was determined by first determining the amount of water required to treat the fabric. The desired treatment rate (2gAV was obtained by mixing 2.4 ml of Deltamethrin with 195 ml of water. This mixture and the cloth required to make the target were placed into an approximately 8 L resealable freezer bag and inverted daily for four days. The cloth bag was removed 1 week after treatment, hung from a fence with clothespins, and allowed to dry. The fuel tank can be placed under the target or next to it. The generated CO_2 flows both through the target and under the bottom edge.

Experimental design. A site selection study was conducted for 16 days (pre-treatment phase) by monitoring sand fly populations at both oases every third night (6 repetitions) with six unbaited (no CO_2) CDC-UV traps (Model 912, John Hock, Gainesville, Florida, USA) per site. During the first night, the CDC traps were evenly dispersed (at least 15 m apart) in the two oases but were operated afterwards from fixed tripods with the trap body suspended 50 cm above the ground. Ultimately, the oasis with the higher sand fly catches and infection rate was chosen as the treated site, and the other oasis became the untreated control.

On day 17, the main study began and was conducted from mid-May to late November 2011. At the treated site, 15 TCT units were evenly dispersed (at least 15 m apart) and operated continuously but no TCT units were placed at the control site. Monitoring every third night with the CDC-UV traps continued at both sites. This regimen was maintained for 2 months. Starting at the third month and continuing for the next 4 months, TCT units at the treated site were reduced from 15 to seven, and monitoring at both sites was reduced to weekly intervals. To compensate for dwindling sand fly catches and to collect sufficient material for dissection, after 1 month, the number of CDC-UV traps used for monitoring was increased from six to 18 at the treated site. To avoid one-sided impact on sand fly population at the treated site, the number of CDC-UV traps also increased to 18 at the control site. CDC traps and TCT units were always placed at least 15 m apart.

A follow-up study was conducted from early March to mid-December 2012. No treatments were applied, but CDC-UV traps were used to monitor sand fly populations and evaluate effects from the previous use of the TCTs on the isolated oasis. The 18 CDC-UV traps were again used at each oasis, but sampling was done every other week. Traps were rotated to avoid positional bias (every third night, and at the end of the study, weekly).

Species identification and infection rates in collected flies. CDC traps were set 1 hour before sunset, and collection nets were recovered at 0600 each morning. Sand flies were transported in cooling bags (5°C) within 2 hours to the laboratory, where they were killed by CO_2 and consecutively processed. Sand flies were identified, counted, segregated by sex, and random samples were taken for species identification and dissection. In the pre-treatment phase, from both sites, 500 females were

dissected. In the post-treatment phase, matching batches of 300 or less females (depending on availability) were dissected from sand flies captured at bi-weekly or monthly intervals.

Dissection was carried out under a stereomicroscope. The guts of the females were examined with a phase contrast microscope for *Leishmania* promastigotes and the heads and genitalia of both sexes (100 females and 100 males from both sites: N=200 per site) were mounted in either Hoyer's or Berlese's medium for species identification (Kravchenko *et al.* 2004). For identification, we used the keys of Artemiev (1980), Lewis and Buttiker (1980, 1982), Lewis (1980, 1982), Lewis *et al.* (1982), and Lane (1986).

Statistics. Sand fly counts were analyzed separately by sex with a generalized linear model (GLM) for a negative binomial regression, using the following model: sand fly count in each trap for the specified time period was dependent on treatment group, trapping interval and the interaction between group and interval. In the original study, data were analyzed in 2-week groupings through week eight. The remainder was analyzed in monthly groupings. In the follow-up study, data were analyzed monthly. A two-tailed Fisher's Exact Test was used to analyze the dissection data using the following model: promastigote infection (yes/no) was dependent on treatment group.. The two-tailed 0.05 significance level was used to determine statistical significance (SAS 2003).

RESULTS

All of the 400 phlebotomine sand flies were identified as *P. papatasi*.

Females collected in 2011. Before the application of the TCT units (pre-treatment), mean CDC trap catches of *P. papatasi* females was 116.4 in what would become the treated oasis compared with a mean of 62.3 in the control oasis ($p < 0.001$) (Table 1 and Figure 2). After application of the TCT units, mean catches of female sand flies decreased significantly within the first 2 weeks to 42.0 ($p < 0.001$) while mean catches in the control oasis increased slightly but not significantly to 79.7 ($p = 0.202$); the difference between the treated and control oases was significant ($p< 0.01$). The average catches in the treated oasis exhibited a significant decline from pre-treatment levels (13.0, 5.4, and 2.3, respectively; $p < 0.001$ for comparisons with pre-treatment). All comparisons of mean catches between treated and control oases were significant at $p < 0.001$ during these three 2-week intervals.

During the last four monthly intervals when only seven TCTs were in operation in the treated oasis, the mean female sand fly catches remained significantly higher than the pre-treatment levels in the control oasis (113.0, 215.6, 288.8, and 136.9, respectively; $p <0.001$ for comparisons with pre-treatment). Meanwhile in the treatment oasis the average catches remained low (1.2, 0.6, 0.7, and 0.3, respectively; $p < 0.001$ for comparisons with pre-treatment levels). All differences between control and treated oases during the last four monthly intervals were significant ($p < 0.001$).

Males caught in 2011. In the pre-treatment period, the mean CDC trap catches of *P. papatasi* males was 88.6 in what would become the treated oasis compared with 52.1 in the control oasis ($p < 0.001$). After application of the TCT units, mean catches of male sand flies decreased within the first 2 weeks to 75.6 ($p = 0.333$). The mean catches in the control oasis increased slightly to 65.7 ($p = 0.159$); the difference between the treatment and control oases was not significant ($p = 0.412$). In the following three 2-week intervals the average male sand fly catches remained significantly higher than the pre-treatment levels in the control oasis (103.7, 133.4, and 119.3, respectively; $p < 0.001$, for all comparisons to pre-treatment). Meanwhile the mean catches in the treated oasis continued a significant decline from pre-treatment levels (55.9, 22.9, and 10.3, respectively; *p*-values for comparisons with pre-treatment = 0.002, < 0.001, < 0.001, respectively). All comparisons of average catches between treatment and control oases were significant ($p < 0.001$) during these three 2-week intervals.

During the last four monthly intervals when only seven TCTs were in operation in the treated oasis, the mean male sand fly catches remained significantly higher than the pre-treatment levels in the control oasis (88.6, 147.8, 170.0, and 72.8, respectively; *p*-values for comparisons with pre-treatment levels were 0.003, <0.001, <0.001, and 0.057, respectively). In the treated oasis the mean catches remained low (2.2, 0.3, 0.0, and 0.1, respectively; $p < 0.001$ for comparisons with pre-treatment levels). All comparisons between control and treated oases during the last four monthly intervals were significant ($p < 0.001$).

Females caught in 2012. In the first 2 months of the follow-up study, trap means were low (4.3 and 4.5 for control and 0.0 for treated) for both oases (Table 2 and Figure 3). From May through October there was a steady rise in the means of the control oasis (49.1, 80.2, 136.8, 81.0, 269.4, and 406.3) followed by declining trap means from November to December (121.5 and 4.6). The pattern was different in the treated oasis with the means remaining low for May through July (0.5, 2.8, and 8.9), rising from August to November (32.8, 157.0, 158.89, and 169.9) and falling to 10.3 in December. Control means were significantly higher than treated means for May through

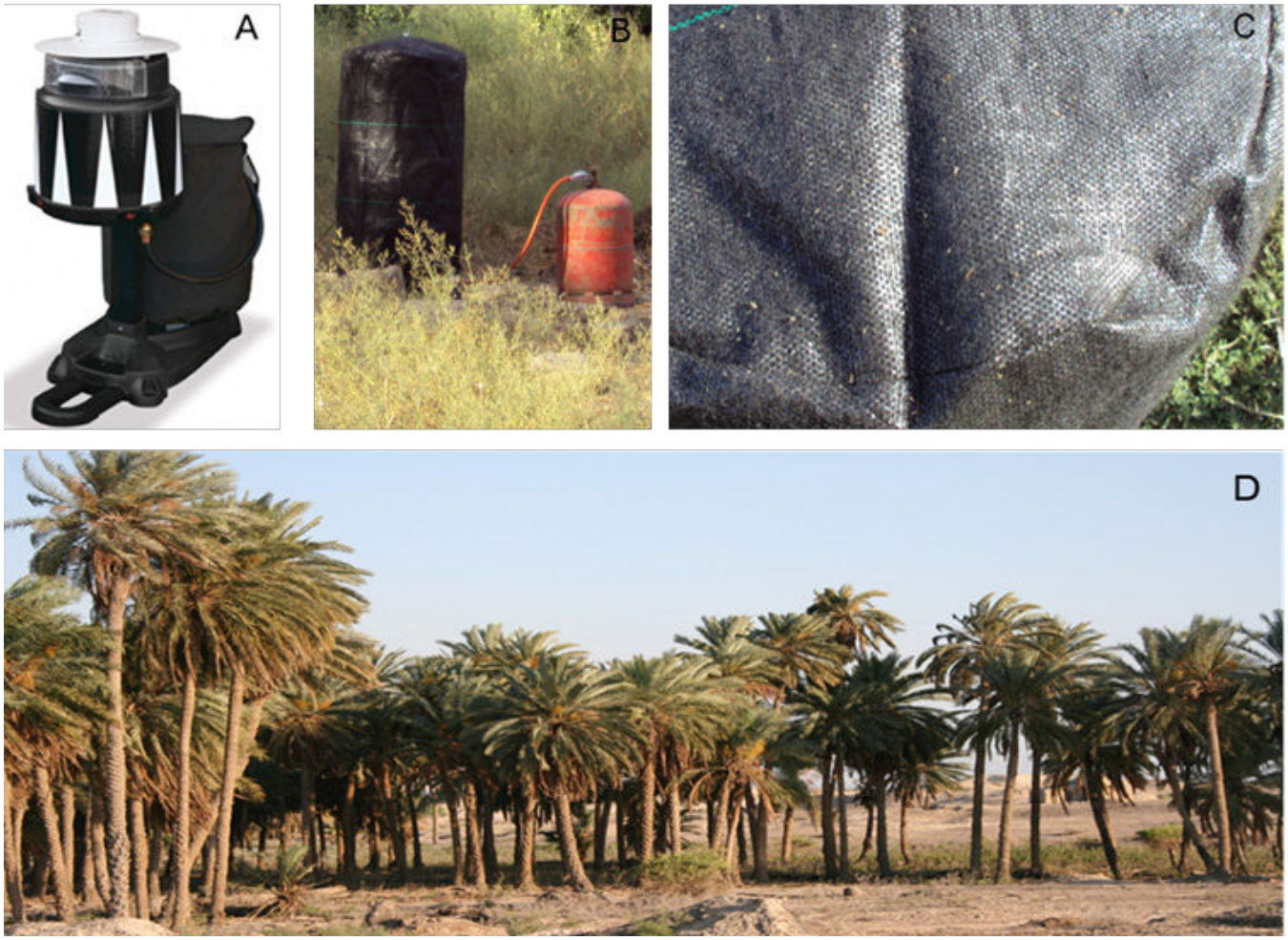

Figure 1. A) Blue Rhino SkeeterVac SV3100 as sold commercially. B) Modified SkeeterVac with TCT. C) Sand flies resting on the textile. D) Overview of experimental oasis.

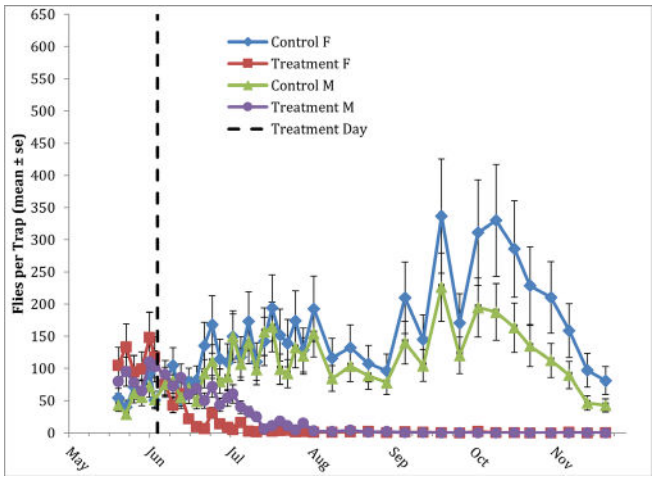

Figure 2. Reduction of male and female *P. papatasi* after application of Toxic Contact Target units in an oasis north of the Dead Sea, May – November 2011.

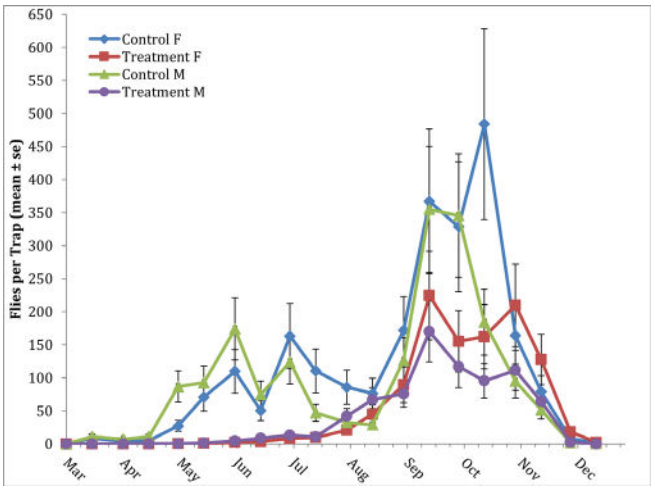

Figure 3. Mean numbers of male and female *P. papatasi* captured in traps in the treated and untreated oases during the follow-up study, March – December 2012.

Table 1. Mean number of male and female *P. papatasi* in traps before and after application of Toxic Contact Target units in an oasis north of the Dead Sea May – November 2011. Treatment starts at week 1. Note: Tx = treatment while C vs. T = control vs. Treated.

	Female					Male				
	Control		Experimental		C vs. T	Control		Experimental		C vs. T
Period	Mean ± se	Pre Tx	Mean ± se	Pre Tx	p	Mean ± se	Pre Tx	Mean ± se	Pre Tx	p
Pre Tx	62.3 ± 8.1	vs. Wk	116.4 ± 15.1	vs. Wk	0.001	52.1 ± 5.8	vs. Wk	88.6 ± 9.7	vs. Wk	0.001
Wks 1-2	79.7 ± 11.3	0.202	42.0 ± 6.0	<0.001	0.002	65.7 ± 8.0	0.159	75.6 ± 9.1	0.333	0.412
Wks 3-4	135.5 ± 19.2	<0.001	13.0 ± 1.9	<0.001	<0.001	103.7 ± 12.5	<0.001	55.9 ± 6.8	0.005	<0.001
Wks 5-6	147.6 ± 20.9	<0.001	5.4 ± 0.9	<0.001	<0.001	133.4 ± 16.0	<0.001	22.9 ± 2.9	<0.001	<0.001
Wks 7-8	157.3 ± 22.3	<0.001	2.3 ± 0.4	<0.001	<0.001	119.3 ± 14.3	<0.001	10.3 ± 1.4	<0.001	<0.001
Wks 9-13	113.0 ± 17.9	0.004	1.2 ± 0.3	<0.001	<0.001	88.6 ± 11.9	0.003	2.2 ± 0.4	<0.001	<0.001
Wks 14-17	215.6 ± 34.1	<0.001	0.6 ± 0.2	<0.001	<0.001	147.8 ± 19.8	<0.001	0.3 ± 0.1	<0.001	<0.001
Wks 18-21	288.8 ± 45.6	<0.001	0.7 ± 0.2	<0.001	<0.001	170.0 ± 22.8	<0.001	0.0 ± 0.0	<0.001	<0.001
Wks 22-25	136.8 ± 21.7	<0.001	0.3 ± 0.1	<0.001	<0.001	72.8 ± 9.8	0.057	0.1 ± 0.1	<0.001	<0.001

Table 2. Mean numbers of male and female *P. papatasi* captured in traps in the control and treated oases during March – December 2012. Note: C vs. T = control vs. treated.

	Female			Male		
	Control	Treated	C vs. T	Control	Treated	C vs. T
Month	Mean ± se	Mean ± se	p	Mean ± se	Mean ± se	P
Mar	4.3 ± 1.2	0.0		5.6 ± 1.4	0.0	
Apr	4.5 ± 1.3	0.0		9.1 ± 2.2	0.3 ± 0.1	<0.001
May	49.1 ± 12.4	0.5 ± 0.2	<0.001	89.8 ± 20.6	0.9 ± 0.4	<0.001
Jun	80.2 ± 20.2	2.8 ± 0.9	<0.001	124.2 ± 28.4	6.5 ± 1.7	<0.001
Jul	136.8 ± 34.4	8.9 ± 2.4	<0.001	85.5 ± 19.6	12.0 ± 2.9	<0.001
Aug	81.0 ± 20.4	32.8 ± 8.4	0.013	30.7 ± 7.2	54.5 ± 12.6	0.081
Sep	269.4 ± 67.5	157.0 ± 39.4	0.129	240.6 ± 54.9	122.9 ± 28.1	0.039
Oct	406.3 ± 101.8	158.8 ± 39.9	0.009	264.9 ± 60.4	106.2 ± 24.3	0.005
Nov	121.5 ± 30.5	168.8 ± 42.4	0.356	73.3 ± 16.8	87.8 ± 20.1	0.577
Dec	4.6 ± 1.3	10.3 ± 2.7	0.038	1.1 ± 0.4	1.3 ± 0.4	0.774

Table 3. Numbers and percentages of promastigote-infected female sand flies in the two oases during the pre-treatment phase and in subsequent weeks after application of the TCT units.

Site	[1]Pre-Treatment	Post-treatment in weeks (W)				
		[2]W 2-4	[2]W 5-6	[2]W 7-8	[3]W 9-10	[4]W 11-26
Treated	57/ 500 11.4%	6/ 300 2.0%	2/ 300 1.0%	0/ 300 0.0%	1/ 245 0.4%	0/ 219 0.0%
Control	33/ 500 6.6%	21/ 300 7.0%	29/ 300 9.7%	19/ 300 6.3%	22/ 245 9.0%	18/ 219 8.2%
P-value	0.009	0.006	<0.001		0.002	

August (< 0.001, < 0.001, < 0.001, and 0.013), higher but not significant in September (0.129), significantly higher in October (0.009), lower but not significantly in November (0.356) and significantly lower in December (0.038).

Males caught in 2012. In the control oasis, trap means for males followed a similar pattern as for females. Means were lower in March and April (5.6 and 9.1) but increased from May through October (89.8, 124.2, 85.5, 30.7, 240.6, and 264.9) and then decreased in November and December (73.3 and 1.1). The same was true in the treated oasis. The means were low from March through July (0.0, 0.3, 0.9, 6.5, and 12.0), higher from August through October (54.5, 122.9, and 106.2), then a decline in November and December (87.8 and 1.3). Trap means in the control oasis were significantly higher than those in the treated oasis from April through July (< 0.001, < 0.001, < 0.001, and < 0.001); in August means from the control oasis were lower but not significantly than those in the treated oasis (0.081); trap means in the control oasis were significantly higher in September and October (0.039 and 0.005) than those in the treated oasis; and in November and December trap means in the control oasis were lower but not significantly than those in the treated oasis (0.577 and 0.774).

Infection rates in collected flies. Before application of the TCT units, the infection rate of the local sand fly populations was higher, 11.4% (57/ 500), in the oasis that became the treated area than in the oasis that became the control area, 6.6% (33/ 500; p = 0.0107). Within the first month after the application of the TCT units, the percentage of infected flies decreased by 88.3% at the treated oasis (57/ 500 versus 8/ 600; p < 0.0001) while it increased by 21.7% at the control oasis (33/ 500 versus 50/ 600; p = 0.3033). In the following 5 months, a single infected sand fly was found at the treated oasis (1/ 764; 0.1%) while at the control oasis 7.7% (59/ 764) of the dissected flies were infected (p < 0.0001).

DISSCUSSION

Although the local *P. papatasi* population was virtually eliminated by the TCT units, it took about a month to achieve this. Two weeks after employing the units, the female population dropped by 76%. After 4 weeks, there was a decrease of 86%, but it took another 4 weeks to reduce the population by 97.5%. Because the TCT were designed to attract host-seeking female sand flies, this delay was even more pronounced with the males (Figs. 2 and 3). An obvious explanation for this phenomenon is the longer (compared to mosquitoes) developmental time of phlebotomine sand flies (Yaghoobi-Ershadi *et al.* 2007). The outcome is a steady supply of young flies over a long period. Adult populations decrease slowly until the kill rate exceeds eclosion rate, even when all adults are being perpetually eliminated.

When a control measure is first implemented, the long reproductive cycle of *P. papatasi* may be a clear advantage for this species. However, after a population has been suppressed, it is difficult for the same population to recover (Hamarsheh *et al.*, 2024). Two months into our study, we removed half of the TCT units, yet the population was not able to recover. It continued to dwindle with time until the mean CDC trap catches dropped below a single female/ male per trap. Simultaneous catches at the control site averaged almost 200 females and 150 males.

The new TCT method not only drastically reduced the number of sand flies, but it also practically eliminated all of the infected females. This is even more impressive if put into context with the size of the collected samples. In the last 5 months of the study, only 874 females were trapped at the treated site. However, during the same period, 86,309 females were trapped at the control site. Using the mean pretreatment infection rate of 7.72%, a total of 6,663 flies in the control site sample were potentially infected.

It appears that this urge for multiple blood meals in combination with a certain lack of hosts resulted in a high contact rate of the sand flies with the TCT units. Apparently almost all females were killed within 4 days, early enough that they could not develop mature infections.

Biting flies, locate vertebrate hosts by responding to their chemical and physical cues (Allan *et al.*, 1987). Chemical cues include CO_2, water vapor, and components of body odor while physical cues are heat patterns and visual stimuli such as color, contrast, shape, and movement (Killick-Kendrick 1999). In the literature, it is well documented that CO_2 is the single most powerful long-distance attractant for female sand flies (Muller *et al.*, 2015) while physical and optical cues are used to further home in for a blood meal (Muller *et al.*, 2015).

In principle, insect control devices must achieve two objectives: 1) attract insects, and 2) kill them. Traps in the classical sense, depend on features like funnel constructions, suction, glue boards, and/or electric grids to remove attracted insects (Service, 1993). However, this is largely wasted effort because a single landing on a pesticide-impregnated target is enough to kill reliably. In some recent published papers (Junnila *et al.*, 2011; Kline *et al.*, 2011a,b) and unpublished experiments, we observed in Israel that mosquito traps, especially in combination with CO_2, attracted female *P. papatasi* from a distance (up to 28

m) but later only some of the attracted flies were removed by the same traps (depending on the trap 9-43%) due to inefficient capture/ killing mechanisms.

In release chambers, we observed that female *P. papatasi* hovered around traps or landed on the traps, far from capture mechanisms. At the same time, sand flies readily and frequently landed on baited, pesticide-impregnated cloth targets, resulting in near total eradication of the female population within a short time.

Attract and kill systems are common practice in agricultural pest control with sound results covering diverse groups such as moths, storage beetles, bark beetles, ants, roaches, house flies, filth flies, snails, and slugs (Olkowski and Daar, 1991; Reuveni, 1995). The concept of attracting and then killing a target species in a relative confined area, rather than treating large stretches of land with few or no vectors, is a great improvement (Day and Sjogren, 1994). Despite the proven success of attract-and-kill systems in agricultural pest control, their application in medical and veterinary entomology remained largely overlooked until recently, even though several documented examples demonstrate their effectiveness (Day and Sjogren, 1994; Kline, 2007). Trials in the mid 80's and 90's to control tsetse flies with attractant-baited, insecticide-impregnated targets (Vale *et al.*, 1995; Willemse, 1991) led to a practical, environmentally friendly and highly efficient method, which is today an important cornerstone in integrated vector control of tsetse flies in Africa (Rayaisse *et al.*, 2010). Attract and kill systems for houseflies, eye gnats, stable flies, and horse flies are becoming increasingly common in US dairies and farms. A growing demand for environmentally friendly products drives this market Geden *et al.*, 2021).

Regarding mosquitoes, there were several attempts to control them by baited traps/ targets. Trials on small islands, with modified conventional mosquito traps and baited pesticide-impregnated targets, were highly successful, and mosquito biting pressures were reduced by more than 90% (Kline and Lemire, 1998; Kline, 2007). On mainland Florida, the same equipment was used for barriers between several residential areas and mosquito breeding sites. The results were less spectacular but offered considerable relief to the local population and some of the former experimental setups were permanently institutionalized (Kline, 2007). In previous studies, it was demonstrated that it is feasible to efficiently attract and kill local mosquito populations with attractive toxic sugar baits (ATSB) in Florida (Muller *et al.*, 2010; Qualls *et al.*, 2014), Israel (Muller *et al.*, 2011), and Morocco (Qualls *et al.*, 2015). In the past, the ATSB method was modified for sand flies and succeeded in several experiments in Israel (Schlein % Muller, 2010) and Mali (unpublished results) to successfully control several different phlebotomine species.

With the right attractants and bait station systems, we were previously able to efficiently attract and control sand flies with the sugar baits, though flies were only attracted from distances up to 5 m. CO_2 is the single most powerful attractant for sand flies and in attraction distance experiments originally designed for sugar baits, we observed attraction to resting humans up to 20 m (CO_2 output about 350 ml/ min) and to CO_2 baited TCT units up to 32 m (CO_2 output about 500ml/ min) (unpublished results of the authors).

The combination of CO_2 as an attractant, generated by the combustion of hydrocarbon, and a pesticide impregnated optical target for the control of sand flies is a simple but new approach. In contrast to conventional traps, no complicated and often inefficient capture/ killing mechanisms are required. A single contact with the target kills the fly.

Residual insecticides applied to surfaces can, in fact, have a repellent effect and may be avoided by target insects and contact with them may be too short or superficial for sufficient toxicity (Coleman *et al.*, 2006, Balaska *et al.*, 2021). In contrast, impregnated, fur-like, cloth targets in combination with CO_2 are explored by females in search for blood meals persistently and for relatively long times (unpublished field observations). In the case of some male sand fly species, hosts or nearby objects are sought out for lecking behavior, a performance in which males await arriving females nearby a suitable host for mating (Chelbi *et al.*, 2011; 2012).

TCT units can be applied in a variety of ways to suit different conditions: they can either be dispersed inside camps and residential areas or they can be arranged as barriers surrounding the area you intend to protect. In principle, TCTs only affect biting flies by "attract and kill", and thus will be less damaging to the ecology than other existing large-area spray treatments for control.

Having our preliminary results in mind, we are convinced that TCTs are a reliable and efficient way to control sand fly populations in large areas with minimum efforts required for maintenance and service.

Further research is needed to see if this new method has an equally high impact on other sand fly species and how the units perform in different types of environments. Though the results of the first trial are very promising, one needs to bear in mind that the existing unit was originally developed for mosquitoes, and it is only reasonable to assume that their performance could be greatly improved if properly adjusted and optimized for sand fly control.

Sound knowledge of behavior and ecology is necessary for such innovative methods, and much future research is needed to exploit vulnerable points in the life cycle/behavior of vectors.

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ASSESSMENT OF SAMPLING METHODS FOR PEAK NALED DEPOSITION DISTANCE FROM FLIGHT LINE DURING AERIAL APPLICATION

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ABSTRACT

Pesticide deposition on natural waters and other surfaces creates a public health risk from their use. The Environmental Protection Agency (EPA) determines deposition with the AGricultural DISPersal model, which does not seem to be an appropriate tool for mosquito control applications. On the other hand, sufficient reliable data on ground depositions from mosquito control sprays to evaluate the estimates from models used by EPA does not exist. This study was conducted to compare different sampling methods to determine peak deposition distance from the flight line when spraying Dibrom® using a helicopter equipped with rotary ULV nozzles. The three replicated tests were conducted at two different sites, one surrounded by trees on all sides and a second site mainly open. At both sites, twenty sampling locations were established, 30.5 m apart, first at 30.5 m and last at 610 m from the flight line. Each location had a spinner mounted on a tripod 1.5 m above ground holding one slide for droplet characterization and one for spray flux, a filter paper attached to a cutting board on the ground, and a petri dish on the same board. Single-pass applications were made at an elevation of 46 m with a helicopter discharging Dibrom at 4.3 L/min, travel speed of 145 km/h and swath of 305 m to deliver Dibrom at 51 mL/ha. Tests on one site were completed in one day, with the first spray an hour before sunset and the last spray an hour after sunset. The droplets on the slides collected during the tests were measured using DropVision system and the amount of Dibrom on slides, filter paper, and petri dish was determined with fluorometry. The droplet size data for all tests and all locations indicate that volume median diameter (VMD) was within the acceptable range of droplet spectrum (9.0 – 22 µm VMD) for ULV adulticide applications. The results indicated a significant effect of sites and samplers on ground deposition. The difference in ground deposition at different distances from the flight line was not significant. The peak ground deposition measured with petri dishes occurred at 91 m from the flight line at the wooded site under low wind and at 520 m in the open field under higher winds. The peak deposition was 33.5% and 49.5% of the application rate at the wooded site and open site, respectively. The results indicated that only the petri dish was able to determine the peak deposition distance from the flight line.

Key words: Health risk, Model, Adulticide, Mosquito control, Spray dispersion

INTRODUCTION

The Environmental Protection Agency (EPA) defines ecological risk assessment as the process of evaluating how likely it is that the environment might be impacted as a result of exposure to one or more environmental stressors, such as chemicals, land-use change, disease, and invasive species (EPA 2023a). The assessment of risk from a chemical pesticide to human health or the environment depends upon the toxicity and the amount of the pesticide to which a person or the environment may be exposed (EPA 2023b). Pesticide deposition on natural waters and other horizontal surfaces is a key input for EPA risk assessments of pesticides. In assessing exposure, mathematical models are used to predict pesticide concentrations in food, water, residential and occupational environments. EPA uses AGricultural DISPersal (AGDISP™) for this purpose, which tracks the movement of spray droplets released into the atmosphere from any aircraft (Barry 1993). Sufficient reliable data on the depositions from aerial mosquito adulticide applications, which can be used to verify risk assessments conducted using AGDISP do not exist. For these verifications, excessive data is required as it is affected by many environmental factors such as wind speed/direction, application parameters such as aircraft type, its elevation and speed, and surface characteristics such as water, soil, or vegetation.

AGDISP™, which was developed by the Unites States Department of Agriculture Forest Service, is a "first-principles" science-based model that predicts spray drift from application sites (EPA 2023b). The model was designed to optimize agricultural spraying operations and has detailed algorithms for characterizing the release, dispersion, and deposition of spray droplets over and downwind of the application area. This model can be used in estimating downwind deposition of spray drift from aerial and ground boom applications. In addition, it is used by the EPA in estimating downwind deposition

of spray drift from forestry and adulticide applications to control mosquitoes.

Agricultural spray applications involve much larger droplets in the range of 100 - 800 µm (Hewitt 2008) than mosquito adulticide applications as mandated by Dibrom label to have volume median diameter (Dv0.5) < 60 µm and 90% of the spray (Dv0.9) < 115 µm. Farooq et al. (2001a; 2001b) have shown significant variation in response to crosswind of up to 15 k/h in wind tunnel by droplets < 100 and > 100 µm in size. In aerial applications, the cross winds are many folds higher than these speeds. The two groups of droplets would certainly respond differently to turbulence, wake, and aerodynamics around aircraft. Settling velocity of the 1- 100 µm droplets in the still air ranges from 3.5 x 10^{-5} to 0.2 m/s (0.007 – 49.2 ft/min) whereas for 100 - 1000 µm droplets it ranges from 0.25 to 3.85 m/s (49.2 – 757.9 ft/min) (Bache and Johnstone 1992). Relaxation time has been defined by Bache and Johnstone (1992) as the time in which a particle adjusts itself to an applied force. The relaxation time of 1-100 droplets is 3.57 µsec to 0.025sec while of 100 - 500 µm droplets, it ranges from 0.025 to 0.204 sec (Bache and Johnstone (1992). For spray droplets in the air, the droplets are subjected to continuously changing airspeed and direction. The relaxation time in this context will be considered as the time droplets take to adjust to their surrounding wind changes (Farooq 2002). With this information, it can be easily imagined that the response of 1-100 µm droplets, >80% of the mosquito adulticides, to wind changes and atmospheric turbulence would be significantly quicker than the response by droplets comprising agricultural sprays. Their significantly small terminal velocity will make them settle very slowly compared to agricultural sprays. Even after coming out of the influence of aircraft wake and downdraft of the rotary wing, the two groups will behave very differently when approaching the ground for deposit. Based on the above discussion, it can be envisioned that AGDISP would overestimate deposition from mosquito adulticide applications.

Many studies have been conducted to evaluate the effectiveness of aerial mosquito adulticide application as a method (Dukes et al. 2004; Lothrop et al. 2007a; Lothrop et al. 2008; Macedo et al. 2010; Chaskopoulou et al. 2011; Burtis et al. 2021; Holcomb et al. 2021; Bibbs et al. 2023). Carney et al. (2008) studied the impact of aerial application of adulticide, pyrethrin, on the number of human West Nile virus (WNV) cases and found that aerial mosquito adulticiding effectively reduced human illness and potential death from WNV infection. Zhong et al. (2004) studied the effect of Dibrom aerial application on honey bees and measured ground deposits using filter papers to collect spray for two hours and analyzed with gas chromatography. They found the highest average Dibrom ground deposition of 2,688 µg/m^2, which resulted in statistically significant bee mortality compared with the controls.

Bargar et. al. (2020) evaluated the mortality and cholinesterase inhibition in butterflies following aerial applications for mosquito control in the National Kee Deer Refuge. Residues of the spray were also determined using filter paper and cotton yarn which determined average residue levels of 2514 µg/m^2 in the target area compared to 736 µg/m^2 in the off-target areas. Butterfly mortality was significantly lower in the non-target area than in the target area. Chaskopoulou et al. (2014) evaluated the effect of ultra-low-volume (ULV) aerial adulticiding of two water-based formulations on non-target organisms and did not find significant non-target mortalities. Rochlin et al. 2022 evaluated the impact of aerial insecticide applications on non-target insects and found that these applications do not pose a significant non-target effect on other insect populations during routine operations.

Hoffmann et al. (2013) compared the droplet size spectrum produced by three nozzles commonly used in vector control in a high-speed wind tunnel when characterized using three different laser-based droplet size measurement systems. Duke et al. 2004 measured ground deposition of the mosquito adulticide fenthion up to 4.83 km downwind by using filter paper when spray was generated with flat-fan nozzles and high-pressure cones. The peak pesticide deposition on the ground determined by gas chromatography was 1,729 and 240 µg/m^2 for the two nozzles, respectively. The highest mean ground deposition from a flat fan nozzle was 742 ± 890 µg/m^2 at 300 m and from a high-pressure nozzle system, it was 106 + 130 µg/m^2 at 1980 m. Lothrop et al. (2007b) measured the deposition of pyrethrins and piperonyl butoxide from aerial adulticide sprays by collecting the spray on filter surfaces spaced up to 300 m on either side of the spray path. The samples were analyzed with high-profile liquid chromatography (HPLC) and found that depositions were not detectable at distances greater than 60 m from the center of the swath.

Currently, sufficient reliable data does not exist to evaluate the estimates from models used by EPA. Also, data are lacking on how this deposition varies in response to wind speed, vegetative cover, and other environmental factors, aircraft elevation, flight speed, or other operational factors or substrates such as water, soil, grass, etc. A preliminary study was conducted to understand different methods to determine peak deposition distance from the flight line when spraying Dibrom using a helicopter equipped with rotary ULV nozzles.

MATERIALS AND METHODS

The tests were conducted at two different sites, one surrounded by trees on all sides while open in the center located at First Coast Technical College, St. Augustine, FL, called the College site (29.93626, -81.36536) (Figure 1) and a second site completely open with no trees in the vicinity, located in Hastings, FL, called the Hastings site (29.69076, -81.50721) (Figure 2). Three replications were made at each site and are called tests 1-3 for the College site and tests 4-6 for the Hastings site. Controls were not included as the objective was to find the distance from flight line where peak deposition occurs and not the absolute deposition. Applications were made as single pass applications at an elevation of 46 m with a Bell 206 Jet Ranger helicopter, perpendicular to the prevailing wind direction. The helicopter was equipped with an ISOLAIR Innovator II model 3900-206ULV spray system with a Shure Flow pump (Isolair Helicopter Systems, Andalusia, Al). The system uses two Micronair AU6539 rotary atomizers (Micron Sprayers Ltd, Bromyard, Herefordshire, UK) with 30 mesh gauze rotating at 9530 rpm. The pressure regulator was set at 200 kPa, and the system was calibrated to discharge Dibrom concentrate (AI: Naled 87.4%, AMVAC Chemical Corporation, Newport Beach, CA) at 4.3 L/min. Flight speed was set at 145 km/h with a swath of 305 m to deliver Dibrom at 51 mL/ha. The spray system produces droplets with a volume median diameter of 36 µm measured at 1.5 m above ground when flying at 12 m above ground. Fluorescent Yellow 131 liquid dye (Milliken & Company, Spartanburg SC) was mixed with Dibrom at 10,000 ppm as a tracer for determination of deposition (Farooq et al 2009). The application conditions were similar to what is normally used by Anastasia Mosquito Control District (AMCD) for adulticiding except at an elevation of 46 m instead of 92 m.

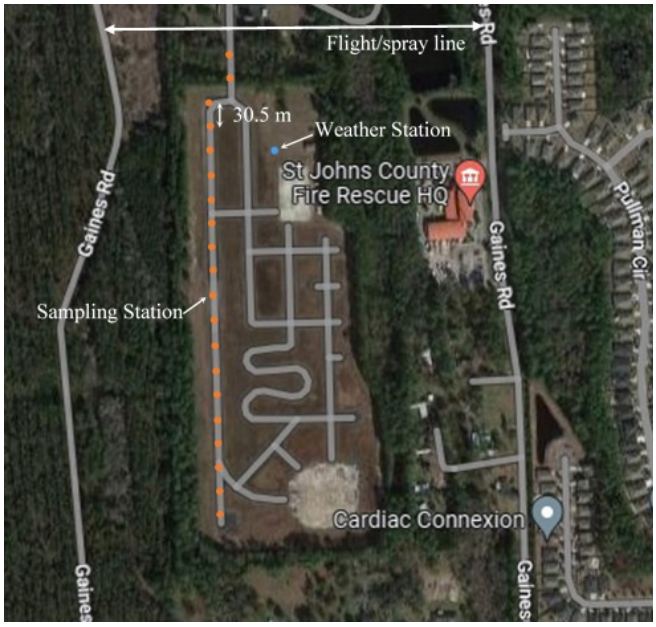

Figure 1. Flight line and sampling layout at College Site.

Figure 2. Flight line and sampling layout at Hastings site.

The sampling layout at the two sites consisted of 20 locations, 30.5 m apart, spread from the first at 30.5 m to the last at 610 m from the flight line. The maximum distance of 610 m was selected as twice the normal swath width for aerial application, as a starting point as this is the first study of its kind. Each sampling location had a spinner mounted on a tripod 1.5 m above ground, a filter paper attached to a cutting board, and a 7 cm inside diameter glass petri dish sitting on the same board. The filter paper and petri dish were 1 m away from the spinner to reduce the impact of the rotating spinner. The spinner was loaded with a Teflon® coated 3 mm slide on one side for droplet size characteristics and a plain 3 mm slide on the other side to collect spray for measurement of spray flux. The sampling line was laid out from a north-to-south direction while the application was made from an east-west direction on the north end of the sampling line. The College site is a driver safety training site with roads and grassy fields. Due to standing water in the grassy fields, the sampling locations were set on the road and two locations had to be staggered (Figure 1). The Hastings site is a grassy airstrip used for aerial sprays for crops. The sampling locations were set on the runway along the length (Figure 2).

Tests on one site were completed in one day, with the first spray an hour before sunset and the last spray an hour after sunset. College site tests were done on October

27, 2022, and Hastings site tests were done on November 14, 2022. All spinners were loaded with slides, and filters and labeled petri dishes were placed on each location when conditions were expected to be suitable for spray. When ready, it was waited for the right conditions and applications were made which lasted about 12 seconds for a path of 490 m, equally distributed on two sides of the sampling line. After the completion of the application, the spray was allowed to settle and pass through the area for 30 minutes, based on a settling velocity of 0.027 m/s for 30 µm droplets, before samplers were collected. Slides for droplets were held upright in foam at the bottom of a box, slides for spray flux and filter paper were stored in pre-labeled re-sealable plastic bags, and petri dishes were covered with respective covers and taped on the sides to keep the covers in place during transportation. After collection, all samples were stored in a dark and cool place. At the end of the day, all samples were moved to the refrigerator and stored until analyzed.

Weather conditions during the trials were recorded using a 3-D ultrasonic anemometer model 81000V, one temperature/relative humidity sensor model 41382VC, and one temperature sensor model 41342VC (R. M. Young Company, Traverse City, MN). The anemometer records three components of air velocity in the x, y, and z direction, wind speed and direction, and wind in the vertical direction. The anemometer and other sensors were set to take 4 readings per second and report data every 30 seconds. The sensor for temperature and humidity was mounted at 9.2 m, an anemometer at 4.6 m, and a temperature sensor at 2.3 m height. The data recorded for two temperatures and wind speed was used to calculate the atmospheric stability ratio with the following equation (Brad 2006):

$$SR = \frac{T_2 - T_1}{U^2} 10^5$$

Where

SR = Stability ratio; < -0.1 Unstable, -0.1 to 0.1 Neutral, and > 0.1 Stable

T1 & T2 = Temperatures at 2.3 and 9.2 m height, respectively in °C

U = wind speed at 4.6 m height in cm/s.

The wind speed and direction variation with time up to 30 minutes after the spray start for tests 1-3 conducted at the College site and for tests 4-6 conducted at the Hastings site is presented in figures 3. The spray operation for each test at the two sites lasted for 25-30 seconds. As shown in these figures, the wind speed was within the range of 1-10 mph during most of the time, with the exception of tests 1 and 2 at the college site and test 4 at Hastings site when it was partially out of the range. However, during the duration of the spray, wind speed was always within the acceptable range. The wind direction shown in the bottom parts of these figures indicates that for the duration of the spray, wind direction was within 20° degrees from the north except for test 2, when it was within 30° from the north. A few times during the waiting time, the wind shifted beyond 30° in tests 1, 5, and 6. However, the time was less than 2 minutes for tests 5 and 6 while it deviated for a total of 10 minutes.

The mean weather parameters of all tests are presented in Table 1. Detailed weather information indicated that the temperature at 2.3 m height was always lower than the temperature at 9.2 m height, which means that conditions during these trials were always stable. This is also demonstrated by the positive stability ratio (SR) during all the tests, as shown in Table 1. The change in SR with time during all tests presented in Figure 3 was positive indicating stable conditions during the spray application and settlement time for all tests. However, it was quite high and variable during tests 2 and 3 shown in the bottom part of Figure 3, mainly due to lower wind speeds during these two tests.

The droplets on the slides collected during the tests were measured using DropVision system (Leading Edge, Fletcher, NC), and all the measurements were completed within 36 hours after the completion of the tests. To measure fluorescent dye on plain slides, the slide in the bag was washed by pouring 20 mL of hexane into the bag and shaking it for 5 minutes. The wash solution was poured

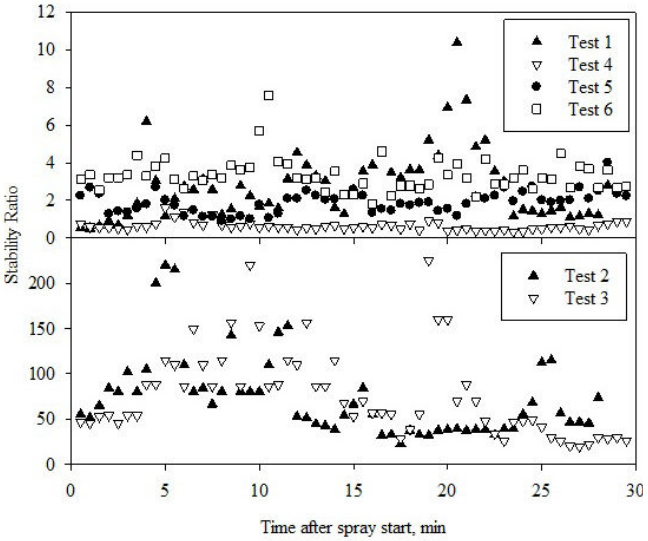

Figure 3. Change in stability ratio with time during spray application and settlement for each test. Test 2 and 3 are plotted separately due to larger magnitude of ratio.

Table 1. Mean weather conditions during aerial spray deposition tests.

Test	Site	Temperature (°C) at		Relative Humidity, %	Wind Speed, km/h	Wind Direction, °	Stability Ratio
		9.2 m	2.3 m				
1	College	21.8	21.4	72.9	4.8	22-28	2.8
2	College	19.4	18.3	79.4	1.6	328-342	73.3
3	College	19.4	18.3	79.0	1.6	336-355	76.7
4	Hastings	20.5	19.7	82.7	12.9	357-28	0.7
5	Hastings	19.5	18.6	88.6	8.7	343-12	1.8
6	Hastings	18.8	17.7	92.9	7.2	337-343	3.0

into the cuvette, and the concentration of dye in the solution was measured using a spectrofluorophotometer (Model RF-6000, Shimadzu Scientific Instruments, Inc. Tampa, FL) and calibrations previously developed with standard solutions. Blank samples containing hexane were used to set the zero reading to eliminate any fluorescence emitted by hexane at used wavelength. The length of each slide was measured, and the surface area was determined. The amount of dye in the solution was converted to µL of Dibrom per square meter of the slide surface using the ratio of dye to Dibrom in the tank and surface area of the slides following procedures explained by Farooq et al. (2009). Dibrom deposits on the filter surface were also determined in the same way except the surface area of the filter paper which was determined using the diameter of the filter paper. Petri dishes were washed by pouring 10 mL hexane and rinsing the inner surface of the Petri dish. The amount of dye was determined the same way as for slides and filter paper. As there was a small variation in the size of petri dishes, the diameter of each petri dish was measured, and the surface area was determined. The deposition of Dibrom in each petri dish was then determined. The deposition on filter paper and petri dishes is called the ground deposition, while deposition on slides is called the spray flux. Both ground deposition and spray flux were expressed as a percent of the applied pesticide rate of 51 mL/ha, assuming that it is uniformly distributed over the whole hectare area.

The deposition data was analyzed using JMP 15.2.0 (SAS Institute Inc, Cary NC) at a 95% level of confidence. The data from each sampler and site was fitted to the normal distribution, and its goodness was tested using the Anderson-Darling test. The normality of the data was also tested using the Shapiro-Wilk test due to the smaller number of data points. All of the data sets were found to be non-normal. Nonparametric one-way analysis was performed with Wilcoxon/Kruskal-Wallis test to test the effects of different sites, samplers, and distances from the flight line on deposition. Means were compared using nonparametric comparisons for each pair using Wilcoxon method.

RESULTS

The droplet size data (Figure 4) for all tests indicate that the volume median diameter (VMD) for all six spray applications ranged from 9.0 – 22 µm and was within the acceptable droplet spectrum for Dibrom of Dv0.5 < 60 µm for aerial applications mandated in the label (AMVACCC 2021).

Overall, the test site significantly affected mean deposition (χ^2 = 29.39, df = 1, p<0.0001), producing more deposition at the Hastings site (2.45 ± 0.20 µL/m^2) than at the College site (2.07 ± 0.26 µL/m^2). Combining the deposition data for the two sites, the deposition on three sampler types was different (χ^2 = 142.21, df = 2, p<0.0001). The slide readings indicated significantly higher deposition (5.34 ± 0.34 µL/m^2) compared to the petri dish (0.73 ± 0.08 µL/m^2) and filter paper (0.75 ± 0.05

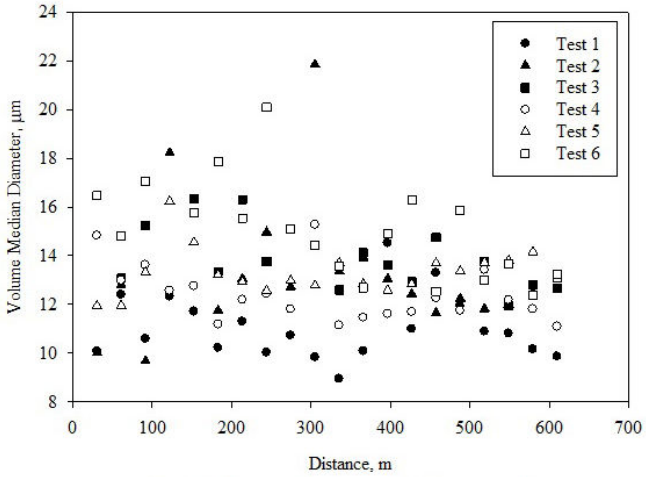

Figure 4. Volume median diameter for all tests at two sites.

Table 2. Mean deposition on three samplers at two sites.

Sampler	Spray deposition (mean ± SE), µL/m²	
	College site	Hastings site
Filter paper	1.28 ± 0.02 A a*	0.21 ± 0.01 B b
Petri dish	0.96 ± 0.07 B a	0.51 ± 0.15 A b
Slide	4.01 ± 0.53** b	6.69 ± 0.33 a

* Means with the same capital letter in the column indicate no significant difference (α= 0.05) among samplers at the two sites. Means with the same small letter in the row indicate no significant difference (α= 0.05) between sites for each sampler.
** Spray flux measured by slides was not compared with the filter paper or petri dish.

µL/m²). At both sites, the difference in mean deposition on samplers was significant (College site: $\chi^2 = 26.70$, df = 2, $p<0.0001$; Hastings site: $\chi^2 = 113.67$, df = 2, $p<0.0001$). The mean depositions on three samplers at the two sites are given in Table 2. As shown in Table 2, between the two sites, there was a significant difference in mean deposition on filter papers ($\chi^2 = 86.90$, df = 1, $p<0.0001$), on petri dishes ($\chi^2 = 42.18$, df = 1, $p<0.0001$), and on slides ($\chi^2 = 12.18$, df = 1, $p = 0.0005$).

Considering the dispersion of spray with distance along the sampling line, the mean deposition was not affected by the distance from flight line at the College site ($\chi^2 = 18.65$, df = 19, $p = 0.47$) as well as at the Hastings site ($\chi^2 = 7.59$, df = 19, $p = 0.99$). At the College site, there was no difference in deposition at distances from flight line on filter papers ($\chi^2 = 14.80$, df = 19, $p = 0.735$), on petri dishes ($\chi^2 = 10.94$, df = 19, $p = 0.926$), and on slides ($\chi^2 = 13.40$, df = 19, $p = 0.819$). Also at the Hastings site, there was no difference in deposition at distances from flight line on filter papers ($\chi^2 = 10.73$, df = 19, $p = 0.933$), on petri dishes ($\chi^2 = 13.71$, df = 19, $p = 0.800$), and on slides ($\chi^2 = 18.28$, df = 19, $p = 0.504$). The spray flux as well as deposition at each distance using filter paper and petri dish at two test sites is presented in table 3.

Table 3. Mean (± SE) spray flux and ground deposition at each distance at two test sites.

Distance from spray line, m	Spray Flux (Mean ± SE), %		Ground Deposition on Filter paper (Mean ± SE), %		Ground Deposition in Petri Dish (Mean ± SE), %	
	College Site	Hasting Site	College Site	Hasting Site	College Site	Hasting Site
30.5	175.8 ± 88.1	108.0 ± 7.0	26.3 ± 0.3	3.6 ± 0.3	17.6 ± 6.7	3.4 ± 1.9
61.0	58.0 ± 41.1	105.5 ± 8.7	26.0 ± 04	3.4 ± 0.3	24.1 ± 4.1	7.0 ± 4.9
91.4	171.1 ± 87.7	101.6 ± 4.8	27.5 ± 1.4	3.0 ± 0.1	33.8 ± 10.5	6.8 ± 4.7
121.9	86.7 ± 40.3	68.2 ± 34.3	25.9 ± 0.6	3.2 ± 0.2	26.5 ± 3.5	77.8 ± 75.1
152.4	70.0 ± 39.0	101.1 ± 8.9	25.8 ± 0.4	2.0 ± 1.0	24.5 ± 4.3	5.2 ± 3.4
182.9	99.7 ± 39.4	120.2 ± 18.3	25.6 ± 0.5	3.6 ± 0.5	21.2 ± 3.0	5.2 ± 4.0
213.4	83.5 ± 30.4	122.1 ± 22.2	25.8 ± 0.3	5.5 ± 2.2	15.9 ± 5.8	3.4 ± 1.5
243.8	61.5 ± 40.1	114.5 ± 11.9	25.5 ± 0.3	5.3 ± 2.1	17.0 ± 6.8	4.1 ± 1.1
274.3	77.9 ± 56.5	130.0 ± 27.3	25.4 ± 0.3	4.9 ± 1.7	15.6 ± 4.8	8.3 ± 2.7
304.8	58.9 ± 42.5	127.1 ± 21.0	25.6 ± 0.5	4.1 ± 1.1	15.3 ± 5.4	10.6 ± 3.8
335.8	56.2 ± 42.4	137.7 ± 23.0	25.7 ± 0.2	3.7 ± 0.7	15.9 ± 5.8	18.0 ± 14.3
365.8	57.5 ± 41.6	142.3 ± 43.9	25.4 ± 0.3	4.7 ± 1.5	16.0 ± 5.8	4.4 ± 1.1
396.2	64.6 ± 49.1	116.9 ± 4.7	26.2 ± 0.1	4.4 ± 1.5	14.9 ± 5.4	4.3 ± 1.0
426.7	55.6 ± 41.4	125.9 ± 16.4	25.5 ± 0.3	3.6 ± 0.5	14.9 ± 5.8	2.7 ± 0.6
457.2	54.9 ± 41.1	142.5 ± 22.6	25.6 ± 0.3	4.1 ± 1.0	16.1 ± 6.5	3.9 ± 0.9
487.7	59.9 ± 42.0	128.5 ± 14.5	25.6 ± 0.4	4.1 ± 1.0	15.5 ± 4.9	7.2 ± 2.4
518.2	57.2 ± 42.5	162.2 ± 52.0	25.4 ± 0.4	4.4 ± 1.4	16.3 ± 6.6	49.8 ± 44.4
548.6	55.7 ± 42.7	162.4 ± 1.7	26.2 ± 0.7	5.1 ± 1.7	16.6 ± 5.2	38.3 ± 33.6
579.1	88.2 ± 74.9	149.3 ± 95.0	20.8 ± 4.9	4.4 ± 1.2	20.3 ± 8.5	4.4 ± 0.3
609.6	54.0 ± 46.3	169.8 ± 46.4	18.1 ± 7.1	4.1 ± 1.1	18.0 ± 8.1	6.3 ± 0.3

The tests at the College site under low wind conditions in the presence of trees resulted (Fig 5) in peak spray flux at 91 m from the flight line determined using slides. The spray flux at that site determined at different distances ranged from 54.6 – 174.7%. The tests at the Hastings site under higher wind conditions in an open area reported (Fig 5) the peak spray flux determined with slides at 580 m from the flight line. The spray flux at that location determined at different distances ranged from 100.6 – 222.6%. Please note that the flux >100% of the applied rate is imaginable due to the two facts. First, the application rate assumes that all the spray is uniformly distributed over the entire hectare, which practically is not the case. Secondly, these spinners, due to their rotation, can collect spray droplets from the surroundings, as reported by Farooq et al. (2014)

The peak ground deposition determined using filter papers and petri dishes at the College site occurred at 91 m from the flight line (Figure 6). The peak ground deposition at this site was more clearly detected by petri dishes than the filter papers, although filter papers generally collected more deposition than the petri dishes. The ground deposition detected using the filter papers at the College site ranged from 17.9 – 27.3%, and deposition detected using petri dishes ranged from 14.8 – 33.5% (Figure 6). At the Hastings site, peak ground deposition determined using petri dishes occurred at 520 m from the flight line. These figures also indicate that filter paper had higher deposition than Petri dishes at the wooded College site but did not detect a peak in deposition. On the other hand, the two samplers indicated similar deposition at most locations at the Hastings site except at the peak deposition distance, where Petri dishes had higher deposition. The ground deposition using the filter papers at the Hastings site ranged from 2.9 – 5.5% and deposition using petri dishes ranged from 2.5 – 49.5%.

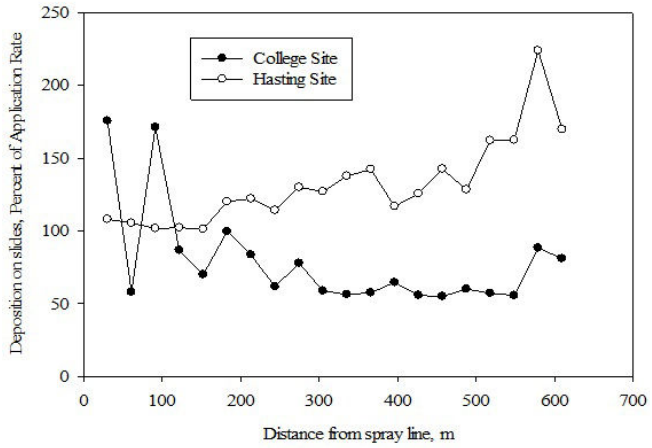

Figure 5. Mean spray flux as percent of application rate at various distances from the spray line at two sites detected by the slides.

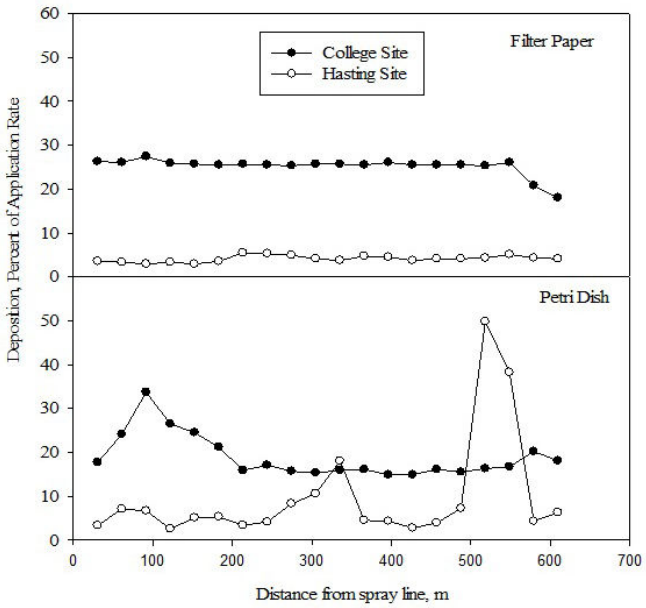

Figure 6. Mean ground deposition as percent of application rate at different distances from spray line at two sites detected by the filter paper (top) and petri dish (bottom).

DISCUSSION

Impact of spray applications on the environment, including human beings, animals, crops, and water has always been a concern. Exposure of these components of the environment to agricultural and vector control pesticides is determined by EPA using the AGDISP model, which tracks the movement of spray droplets released into the atmosphere from any aircraft (Barry 1993). AGDISP was developed to predict spray drift, mainly from agricultural application sites, but it is now used for predicting the deposition of adulticide applications to control mosquitoes (EPA 2023b). Spray droplets in the air are subjected to continuously changing airspeed and direction. Significant differences in response to crosswind by mosquito control sprays and agricultural sprays have been reported (Farooq et al. 2001a; 2001b). Aerial applications of these two spray types respond differently to turbulence, wake, and aerodynamics around the aircraft. There is also a considerable difference in the settling velocity and relaxation time of the droplets comprising mosquito and agricultural sprays (Bache and Johnstone 1992; Farooq 2002).

Based on the above discussion, it can be safely predicted that AGDISP in its current form is not a suitable tool to estimate deposition from mosquito adulticide

applications, while sufficient empirical data also does not exist to compare estimates from models used by EPA. This study focused on finding methods suitable to determine peak deposition distance from the flight line when spraying Dibrom using a helicopter equipped with rotary ULV nozzles.

The spray flux (Figure 5) and ground deposition determined using petri dishes (Figure 6) at the College site peaked at 91 m from the flight line. At the Hastings site, spray flux (Figure 5) and ground deposition (Figure 6) peaked at 580 m and 520 m, respectively, from the flight line. The wind speed at the College site was generally low and the site was surrounded by trees. There was a row of trees between the sampling grid and the flight line. It is known that the tree line restricts the movement of air resulting in a dead spot on the downwind side of and close to the tree line (Heisler and Dewalle 1988). The tree line also creates relatively strong air movement toward the ground at the leeward side of the tree row (Farooq et al. 2023). That structure may have pushed the spray released above the trees to the space after the trees where the three sampling locations covering 91 m were present. Also, the falling spray moved slowly in the horizontal direction due to wind movement restriction by the trees and resulting vortices behind the trees. The detailed wind speed data during all the tests shows that wind speed during tests 1, 4, 5, and 6 was well within the range of the acceptable wind speed for adulticiding. However, wind speed during tests 2 and 3 was within range during application which lasted only 12 seconds, but during wait times, the wind at times was less than 1.6 km/h which may have helped the spray descend faster, especially when the spray plume was under the influence of air generated by propellers of the helicopter. This slow movement caused the spray to stay longer in the area resulting in more deposition on filter paper compared to the Hastings site (Figure 6 top) by settlement. Part of the spray might have been trapped in the air moving above trees (Farooq et al. 2023) and risen up into the atmosphere. It is also possible that some of the spray might have been intercepted due to impaction on to the tree canopy between the spray release point and sampling lines. Due to these two factors, the spray flux recorded at the College site was less than that recorded at the Hastings site (Figure 5). At the Hastings site, wind speed was considerably high which quickly pushed the spray through the grid. This faster movement did not leave enough time for the spray to settle resulting in low deposition on filter papers or in petri dishes. The stronger wind also did not let the spray mix well into the environment and thus, the spray moved as a concentrated cloud resulting in higher spray flux determined with slides at this location.

The results of this study indicate that petri dish may be a suitable method to determine peak ground deposition distance from the flight line whereas slides could be used to determine the peak of spray flux at a height from the ground. The results also indicate the difference in distance where the peak deposition occurs between a field surrounded by trees and an open field.

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RESISTANCE IN *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* TO COMMERCIAL PYRETHROID INSECTICIDES AQUALUER®20-20 AND DUET®

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ABSTRACT

Insecticide resistance is a global issue in effective mosquito control. Since only a limited number of insecticide classes are available to mosquito control programs, reliance on a few active ingredients has developed resistance in many mosquito species. Several studies have been published documenting the development of resistance to pyrethroid active ingredients in Florida populations of *Aedes aegypti* (Linnaeus). However, there are only a few published studies on resistance levels of *Aedes* species to formulated products used in mosquito control programs. This study was conducted to determine baseline laboratory resistance levels of different populations of *Ae. aegypti* and *Aedes albopictus* (Skuse) in St. Johns County (SJC), Florida to Aqualuer 20-20® and Duet®. *Ae. aegypti* and *Ae. albopictus* were collected separately from five mosquito control zones where they received different treatment pressures of insecticides. Topical application bioassays were conducted on those populations to determine the doses required to achieve 100% population mortalities and resistance ratios compared to a susceptible population of each species. Based on the availability of mosquitoes, the Centers for Disease Control and Prevention (CDC) bottle bioassays were conducted on the same populations. All three *Ae. aegypti* populations tested were detected as having high resistance to at least one formulation, irrespective of the insecticide treatment pressure. The only *Ae. albopictus* population to display resistance came from the highest insecticide-pressured mosquito control zone and this is the first quantification of high pyrethroid resistance (RR>10) in *Ae. albopictus* in Florida. The study established the baseline laboratory resistance levels of *Ae. aegypti* and *Ae. albopictus* in SJC to the two pyrethroid formulations. Results indicated the limited efficacy of the synergist piperonyl butoxide (PBO) in reversing high insecticide resistance and highlighted the need for routine monitoring in different populations in the target area.

Key words: insecticide resistance, *Aedes aegypti*, *Aedes albopictus*, formulated products, synergist

INTRODUCTION

Aedes aegypti (Linnaeus) and *Aedes albopictus* (Skuse) are vectors of arboviruses such as dengue virus, yellow fever virus, chikungunya virus, and Zika virus (Jansen et al. 2010, Gubler 2011, Higgs and Vanlandingham 2015, Kauffman and Kramer 2017). These species are known to be invasive (Juliano et al. 2005, Baldacchino et al. 2015) and distributed in many different geographic regions worldwide (Kraemer et al. 2015, Laporta et al. 2023). Both species are widespread in Florida with recorded presence of *Ae. aegypti* and/or *Ae. albopictus* in 56 out of 67 counties (Parker et al. 2019). Due to many constraints vector control has become the primary option (WHO 2009) to mitigate disease transmission and it is accomplished mainly through integrated mosquito management (IMM) (CDC 2022). The most widely used control method for *Aedes* populations is the application of chemical insecticides (Gilkes et al. 1956, Manjarres-Suarez and Olivero-Verbel 2013). However, the repeated use of insecticides has posed the risk of resistance development (Ranson et al. 2010). Dichlorodiphenyltrichloroethane (DDT – an organochlorine) was the first chemical used to control *Ae. aegypti*, which later became ineffective due to the development of resistance (Manjarres-Suarez and Olivero-Verbel 2013). After the detection of high levels of widespread resistance against organophosphates and carbamates (Rawlins and Wan 1995, Tikar et al. 2009, Manjarres-Suarez and Olivero-Verbel 2013) the choice of insecticides for the control of *Aedes* species was pyrethroids. Over the years, the use of pyrethroids against *Aedes* species has been gradually increasing (WHO 2011, Smith et al. 2016) and the reliance on a few registered pyrethroid active ingredients (AIs) has led to the development of pyrethroid resistance in *Ae. aegypti* and *Ae. albopictus* (Smith et al. 2016, Moyes et al. 2017, Amelia-Yap et al. 2018). Pyrethroid resistance in *Ae. aegypti* has high development rates and is relatively common in many countries including the USA (Ahmad et al. 2007, Fonseca-González et al. 2011, Cornel et al. 2016, Amelia-Yap et al. 2018, Demok et al. 2019, Kandel et al. 2019, Yang et al. 2020, Hernandez et al. 2022, Asgarian et al. 2023). The

levels of resistance in *Ae. albopictus* appears relatively low at present compared to *Ae. aegypti* (Liu et al. 2004, Vontas et al. 2012, Estep and Sanscrainte 2024). Resistance to pyrethroids in Florida populations of *Ae. aegypti* is well documented and indicated that most populations have developed high resistance (Estep et al. 2018, McAllister et al. 2020, Parker et al. 2020, Schluep and Buckner 2021, Lucas and Bales 2022). *Ae. albopictus* populations show slow rates of resistance development with susceptibility or low resistance in most tested populations (Liu et al. 2004, Waits et al. 2017, Parker et al. 2020, Estep and Sanscrainte 2024).

The establishment of resistance in a population could take time depending on the amount of selection pressure (Shi et al. 2015), population characteristics (Fotakis et al. 2017), and environmental conditions (Corbel et al. 2019). The development rates and acquired resistance levels for the same insecticide can vary between sub-populations of a species even on small geographic scales (Fonseca-González et al. 2011, Ocampoa et al. 2011, Shin et al. 2012, Deming et al. 2016, Richards et al. 2018, Mundis et al. 2020, Scott et al. 2021, Stoops et al. 2023). Furthermore, the resistance levels of the same population can be different between an active ingredient (AI) and a formulated product (FP) with the same AI (Richards et al. 2018, Scott et al. 2021). Differences in resistance levels in sub-populations of mosquitoes within a treatment zone may not be suitable for a single control strategy. Therefore, understanding the resistance profiles of different populations for different AIs and corresponding FPs used in the current control program is vital for effective control operations. Resistance to pyrethroid AIs in *Ae. aegypti* and *Ae. albopictus* in St. Johns County (SJC), Florida was reported by previous studies (Waits et al. 2017, Wang et al. 2023). The present study was conducted to establish baseline resistance levels of different populations of *Ae. aegypti* and *Ae. albopictus* in SJC to pyrethroid FPs used in the IMM program of the Anastasia Mosquito Control District (AMCD) which is responsible for mosquito control in the county.

MATERIALS AND METHODS

Study area

The study was conducted in SJC, where mosquito control is administered by the AMCD adhering to an integrated approach. The distribution of *Ae. aegypti* in SJC is restricted primarily to the coastal belt while *Ae. albopictus* spread across the county (Aryaprema et al. 2024). Five adjacent mosquito control zones within and around the coastal belt (Fig. 1) were selected to collect both species from the same locations. The mainly residential and commercial C01 zone (Anastasia Island) is almost isolated from other zones by the inter-coastal waterway. The C02 zone comprises downtown St. Augustine and two adjacent residential/commercial areas. The C06 zone is mainly residential while the C10 zone has residential premises and conservation lands. The Cemetery zone refers to Evergreen Cemetery which lies in the C06 zone towards the C02 zone. Hereafter in this text, the populations of *Ae. aegypti* and *Ae. albopictus* in those control zones are referred to with the zone name and the species name linked by a hyphen (e.g. C01_*Ae. aegypti*, Cemetery_*Ae. albopictus*). The five zones receive different pyrehroid treatment pressures based mainly on the mosquito-borne disease risk and nuisance level. Based on the number of treatments per year and the size of the zone, the C01 zone has received the highest pressure over the last few years. The Cemetery does not receive deliberate insecticide treatments but may be subjected to insecticidal drifts from adjacent zones and was considered the least insecticide-pressured zone.

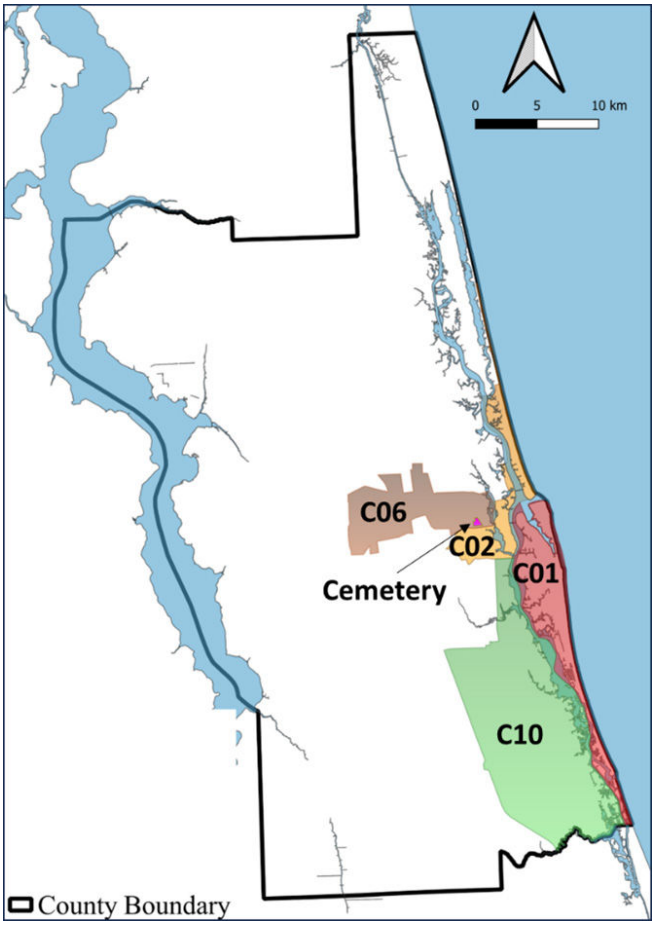

Figure 1. Location of the five selected mosquito control operational zones in St. Johns County, Florida

Mosquito collection and rearing

Aedes eggs were collected separately from the five zones using ovitraps during the 2022/2023 mosquito seasons. Ovitraps consisted of black plastic containers that were lined with a piece of seed germination paper (Anchor Paper Co., St. Paul, Minnesota) that served as mosquito oviposition substrate (ovipaper), and were filled halfway with a diluted hay infusion). Infusion and ovi-papers were replaced weekly, the collected ovi-papers were brought to the AMCD laboratory on dampened kitchen towels and were allowed to dry overnight. Eggs were counted to determine viability and stored in plastic containers with a piece of dampened sponge. Once sufficient numbers for bioassays were collected, eggs of different zones were hatched and reared separately in the AMCD insectary which is maintained at 26±2°C, 80±% relative humidity, and a constant 14-h-light:10-h-dark cycle. Alongside the batches of pyrethroid susceptible colonies of *Ae. aegypti*, 1952 Orlando strain (Pridgeon et al. 2008) and *Ae. albopictus*, 2003 Gainesville strain (Jiang 2022) were reared to be used as references in respective tests. Larvae were fed with Tetramin® fish powder (Tetra GMBH, Germany) and the emerged adults were provided with 10% (wt/vol) sucrose solution ad libitum. F1 generation adults were separated into *Ae. aegypti* and *Ae. albopictus* populations and tested if the numbers were sufficient for testing or continued rearing up to F2 (F0=field population). The rearing of populations was not continued beyond the F2 generation as successive generations can either mask or overestimate the resistance levels in a population (Xu et al. 2014, CDC 2016).

Determination of insecticide resistance levels

AMCD has been using two pyrethroid FPs, Aqualuer®20-20 (20.6% permethrin + 20.6% PBO) and Duet® (5% Sumithrin + 1% prallethrin + 5% PBO) for more than 10 years in its IMM program. Those two FPs were selected to determine the resistance levels of different populations of *Ae. aegypti* and *Ae. albopictus* in SJC. Topical application bioassays (TAB) were used to determine the AI dose in FP required to achieve 50% population mortality at a given time (LD_{50}) and resistance ratios at LD_{50} (RR_{50}). Based on the availability of mosquito samples, the Centers for Disease Control and Prevention (CDC) bottle bioassays (CDCBB) were conducted. Aqualuer 20-20 TABs were conducted with the mosquitoes collected in 2022 and Duet TABs and all CDCBBs were conducted with the mosquitoes collected in 2023.

Topical application bioassays

Both field and reference populations were tested with TABs (WHOPES 2006). The two FPs (Aqualuer 20-20 and Duet) were diluted in acetone to prepare 5-6 concentrations appropriate for each population to cause 0% to 100% mortality. The AI dose (permethrin in Aqualuer 20-20 and sumithrin in Duet) of each concentration was calculated based on the label information. Acetone was used as the negative control. Depending on the availability, each dose was tested with 10 - 20 nonblood-fed female mosquitoes (5-7 days post-emergence). A 0.2 µL droplet of a dose was applied to the dorsal thorax of a CO_2 anesthetized female mosquito on a 4°C chill table using a repeating dispenser with a 10 µL syringe (Hamilton Company Inc. Nevada, USA), and mortality was recorded at 24 h post-treatment. The average weight of a mosquito in each TAB was determined by weighing 50 mosquitoes to calculate the AI dose received per mg of a mosquito. Each TAB was replicated 3-4 times for each population. Any replicate with >10% control mortality was discarded and the test was repeated. There were no replicates with control mortality between 3-10%, thus not requiring mortality corrections (Abbott 1925). LD_{50} was determined using Probit analysis of SPSS (IBM® SPSS® Statistics, Version 20). The goodness of fit of the Probit model was checked for significance ($P>0.05$). If the model fit was not significant, the results were discarded and the test was repeated, based on the availability of mosquitoes. LD_{50} was considered significantly different if there was no overlap of 95% confidence intervals (CI) (Marcombe et al. 2014). RR_{50} of each field population was determined by the LD_{50} of the field population compared to that of the reference population (Estep et al. 2018). Resistance levels were classified as high resistance ($RR_{50}>10$), moderate resistance ($5<RR_{50}<10$), and susceptible ($RR_{50}<5$) (WHO 2016).

The Centers for Disease Control and Prevention bottle bioassay

Based on the availability, two field populations were re-tested with the modified CDCBB to accommodate formulated products (Petersen 2003, CDC 2023) alongside respective reference populations to compare with the TAB results. CDCBBS of reference populations were conducted with FPs and corresponding AIs. Field populations were tested only for FPs as the unavailability of mosquito samples prevented AI testing. Six glass Wheaton® bottles (250 ml capacity) were prepared for each assay; 2 control bottles with acetone and 4 test bottles with the insecticide (AI or FP). Control bottles were treated with 1 ml acetone and test bottles were treated with 1 ml of insecticide solution at the CDC recommended diagnostic dose for each AI (permethrin: 43 µg/ml and sumithrin: 20 µg/ml. Although there are two AIs in Duet only sumithrin was selected for testing

as it is the primary killing AI in the formulation. The materials provided with CDCBB kits were used in the AI tests. The AI doses of FPs were calculated based on the label information to match the respective CDC diagnostic doses. Fifteen to twenty-five nonblood-fed females (5-7 days post-emergence) were introduced into each bottle and mortality counts (number of mosquitoes that cannot fly or stand) were taken every 5 min until 15 min and every 15 min thereafter until 2 h or until all mosquitoes were dead. Mosquitoes were then transferred to paper cups with mesh covers, provided sugar water, and left in a temperature and humidity-controlled incubator to take 24 h post-treatment mortality counts. The diagnostic time (DT) of each AI and FP was determined as the time taken to achieve the 100% mortality of the reference population. A population was considered resistant if it exceeded the DT to achieve 100% mortality. Resistance status was classified by the percent mortality of the field population at the DT: <90% mortality=resistant, 90-97%=developing resistance, >97% mortality=susceptible (CDC 2023). The 24 h mortality counts were used to monitor the recovery of mosquitoes which will indicate the possible presence of the knockdown resistance (*kdr*) mechanism (CDC 2023).

RESULTS

Topical application bioassays

Aqualuer 20-20 TABs for *Ae. aegypti* were conducted only on the C02 population. The LD_{50} of Aqualuer 20-20 for the reference *Ae. aegypti* population was 0.008 ng/mg while that was 0.64 ng/mg for the C02_*Ae. aegypti* population. Thus, the C02_*Ae. aegypti* was 80-fold resistant (RR_{50}=80) to Aqualuer 20-20 compared to the reference population (Table 1). Two field populations, C02_*Ae. aegypti* and C01_*Ae. aegypti*, were tested for Duet. The LD_{50} of Duet for the reference population was 0.277 ng/mg while LD_{50}s for the C02_*Ae. aegypti* and C01_*Ae. aegypti* were 18.108 and 28.462, respectively. Both populations have acquired high resistance to Duet with 65-fold (*C02_Ae. aegypti*) and 103-fold (C01_*Ae. aegypti*) resistance compared to the reference population (Table 1). The Duet LD_{50} of the C01_*Ae. aegypti* was significantly higher than that of the C02_*Ae. aegypti* (95% CIs not overlapping).

Four *Ae. albopictus* populations (from Cemetery, C10, C06, and C01 zones) were tested with TABs. The LD_{50} of Aqualuer 20-20 for the reference population of *Ae. albopictus* was 0.038 ng/mg while it was 0.026 ng/mg for the Cemetery *Ae. albopictus* population. The RR_{50} of the Cemetery *Ae.albopictus* population was less than 1 indicating its susceptibility to Aqualuer20-20. The C10_*Ae. albopictus* and C06_*Ae. albopictus* (LD_{50}: 0.071 and 0.084 ng/mg respectively) indicated susceptibility with RR_{50}<2.5. The model fitness of the C10_*Ae. albopictus* with Aqualuer 20-20 was not significant (P=0.04) and the LD_{50} had a broader 95% CI (Table 1). However, the model to establish the baseline resistance level was accepted due to the unavailability of mosquitoes to repeat the test. The Aqualuer 20-20 LD_{50} of the C01_ *Ae. albopictus* (1.574 ng/mg) was 41-fold higher than the reference population (0.038 ng/mg), indicating high resistance, and was significantly different from all other populations. Duet LD_{50}s for the Cemetery, C10, C06, and C01 zones for the field *Ae. albopictus* populations were 0.599, 1.043, 0.716, and 0.852 ng/mg respectively. All four populations showed susceptibility to Duet compared to the reference population (LD_{50}: 0.535 ng/mg) with RR_{50}<2. The C10_*Ae. albopictus* required a significantly higher LD_{50} than the Cemetery *Ae. albopictus* and C06_*Ae. albopictus.*

Table 1. Lethal doses and Resistance ratios of field *Aedes aegypti* and *Aedes albopictus* populations to Aqualuer®20-20 and Duet® determined by topical application bioassays

Species	Population	Aqualuer 20-20		Duet	
		LD_{50} (95% CI)	RR_{50}	LD_{50} (95% CI)	RR_{50}
Aedes aegypti	Reference	0.008 (0.005 - 0.013)	X	0.277 (0.235 - 0.329)	X
	C02	0.640 (0.475 - 0.836)	80	18.108 (12.422 - 34.703)	65.37
	C01	X	X	28.462 (19.205 - 55.205)	102.75
Aedes albpoictus	Reference	0.038 (0.024 - 0.063)	X	0.535 (0.468 - 0.608)	X
	Cemetery	0.026 (0.014 - 0.049)	0.68	0.599 (0.483 - 0.736)	1.12
	C10	0.071 (0.007 - 2.528)	1.87	1.043 (0.901 - 1.202)	1.95
	C06	0.084 (0.041 - 0.191)	2.21	0.716 (0.594 - 0.857)	1.34
	C01	1.574 (0.543 - 9.987)	41.42	0.852 (0.712 - 1.016)	1.59

LD_{50} : insecticide dose required for 50 % mortality of the population
RR_{50} : resistance ratio at 50% mortality
X : data not available

CDC bottle bioassays

CDCBBs with FPs and corresponding AIs were conducted on the two reference populations. Only two field populations, the Cemetery *Ae. aegypti* and the C01_*Ae. albopictus*, were tested with CDCBB and both populations were tested only for FPs.

The DT of permethrin for the reference *Ae. aegypti* was 30 min as expected (Scott et al. 2021) and it was reduced to 10 min with Aqualuer 20-20. However, the Cemetery *Ae. aegypti* had only 27% mortality with Aqualuer 20-20 at the DT. It took 75 min for 100% mortality, with some mosquitoes bouncing back to give only 44% mortality at 24 hr (Fig. 2A). Similarly, the 15 min DT of sumithrin for the reference *Ae. aegypti* was reduced to 10 min with Duet. The Cemetery *Ae. aegypti* had only 56% mortality with Duet at the DT which took 45 min for 100% mortality. According to the CDC classification of resistance status (CDC-CONUS 508), the Cemetery *Ae. aegypti* population was resistant to both Aqualuer 20-20 and Duet. The presence of a high rate *kdr* mechanism was suggested by the 50% recovery at 24 hr (Fig.2B).

The 30 min DT of permethrin for the reference *Ae. albopictus* population was reduced to 10 min with Aqualuer 20-20. It required 45 min to achieve 100% of the C01_ *Ae. albopictus*. There was only 90% mortality at the DT indicating that the population is developing resistance. Similarly, the sumithrin DT of the reference *Ae. albopictus* was 45 min (99% mortality at 30 min) and it was reduced to 5 min by Duet. At 5 min, C01_*Ae. albopictus* had only 94% mortality indicating the population was developing resistance to Duet. The recovery at 24 hr was less than 2% in both tests.

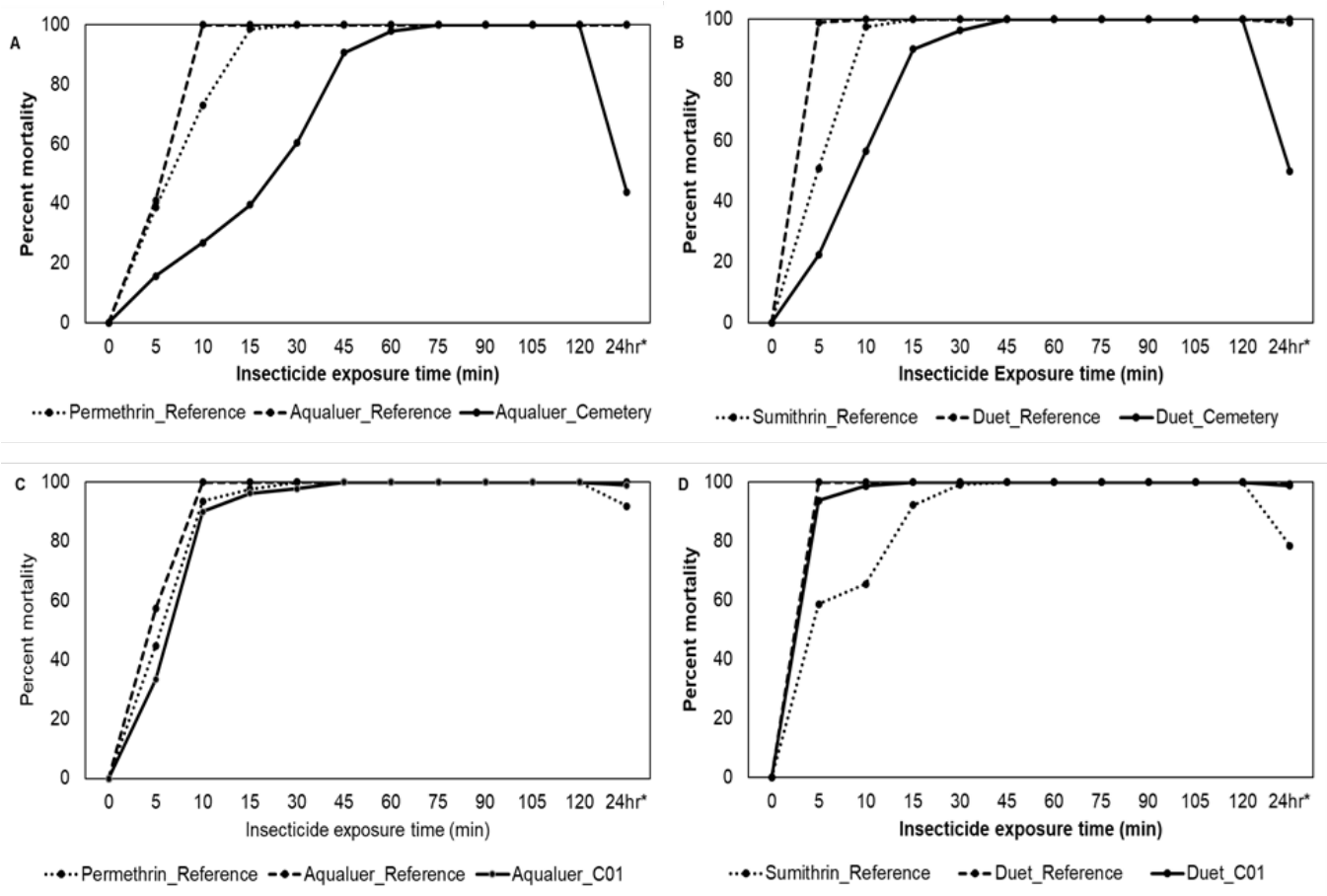

Figure 2. CDC bottle bioassay results of *Aedes aegypti* in the Cemetery zone (A and B) and *Aedes albopictus* in the C01 (C and D) with Aqualuer 20-20 and Duet

DISCUSSION

Routine monitoring of insecticide resistance has become pivotal in mosquito control programs. Resistance levels are often determined for the technical grade AIs used in insecticides. However, operational insecticides are products of AIs formulated with other ingredients some of which are synergists. Those synergists improve the effectiveness of insecticides by inhibiting certain resistance mechanisms. Therefore, monitoring resistance with formulated products used in routine control operations is a required tool. This study attempted to establish baseline resistance levels of small-scale geographical populations of *Ae. aegypti* and *Ae. albopictus* in SJC for two formulated pyrethroids in our IVM program. TABs were conducted to compare resistance levels of different operational zones that receive different treatment pressures.

Resistance to pyrethroid AIs in *Ae. aegypti* is common in Florida populations (Estep et al. 2018, Parker et al 2020, Lucas et al. 2024). However, evidence of resistance for formulated pyrethroids is documented only for a few populations (Scott et al. 2021, Lucas and Bales 2022). In the present study, resistance was detected in *Ae. aegypti* to Aqualuer 20-20 (permethrin + PBO) in the C02 (TAB) and the Cemetery (CDCBB) mosquito control zones in SJC. Both C01_*Ae. aegypti* and C02_*Ae. aegypti* were resistant to Duet and resistance levels of the two populations demonstrated the effect of insecticide treatment pressure on the development of resistance. Although the unavailability of samples prevented cross-checking with the alternative test the results were accepted as all the tested *Ae. aegypti* populations, including the least pressured Cemetery *Ae. aegypti*, showed high pyrethroid resistance. Previous studies reporting permethrin

resistance in *Ae. aegypti* in SJC (Wang et al. 2023) and widespread pyrethroid resistance in *Ae. aegypti* in Florida (Parker et al. 2020) support our results. The high rate of mosquito recovery at 24 h with both FPs indicates the phenotypic expression of the *kdr* mechanism in C01_*Ae. aegypti* and C02_*Ae. aegypti* (CDC 2023).

There was a discord in results between the TAB and CDCBB on C01_*Ae. albopictus* with Aqualuer 20-20. which could be attributed to differences between the two methods; the CDCBB depends on a time threshold with a predetermined dose whereas the TAB depends on a dose-response analysis, the CDCBB mortality counts conclude at 2 h which may be falsely representing higher mortality for a population with high *kdr* whereas the TAB may illustrate a more accurate representation of true mortality by counting at 24 h, in the CDCBB the dose of insecticide received by mosquitoes is unknown and depends on the tarsal contact time whereas the TAB assesses the dose per milligram of mosquito via direct application of a range of predetermined doses. Furthermore, the DT determined in our study was an approximation as there was a steep increase in mortality from 60% -100% between 5 min to 10 min (Fig. 2C). If the actual DT was less than 10 min the 100% mortality of the C01_*Ae. albopictus* at DT would have been <90% and the population would have been categorized as resistant. Also, an almost one-year time gap between the conduction of the two methods warrants consideration as the treatment pressures during the two periods could be different, possibly creating genetic variation for insecticide resistance in the population. A previous study reported discordance between the results of the two test methods on *Ae. albopictus* in SJC (Waits et al. 2017). Althof and Huijben (2022) demonstrated the higher power of TAB over CDCBB to differentiate between resistant and susceptible populations and assess changes over time and between populations. As such, it was decided to establish the laboratory baseline of C01_*Ae. albopictus* as resistant to Aqualuer 20-20. The C01_*Ae. albopictus* being the highest insecticide-pressured population of the tested populations supports our decision. The assumed geographical isolation of the C01_*Ae. albopictus* might have played a role in the resistance development in *Ae. albopictus*. We believe this is the first documentation of strong pyrethroid resistance in *Ae. albopictus* in Florida (Estep AS, Sanscrainte ND. 2024). However, the almost negligible recovery at 24 h indicated that the *kdr* may not be the main attributing mechanism for permethrin resistance in C01_*Ae. albopictus*. However, further investigation is required to confirm the resistance status and determine the major mechanism of resistance. The RR_{50}s of *Ae. albopictus* in all other zones were <3 fold indicating susceptibility to low-level resistance (Estep and Sanscrainte 2024) to both FPs. The two methods agreed when the populations were susceptible or had low-level resistance. Our results agree with the fact illustrated by Parker et al. (2020) that Florida populations of *Ae. albopictus* demonstrated more variability than *Ae. aegypti* in insecticide susceptibility to pyrethroid AIs. Although the pyrethroid resistance in *Ae. albopictus* has been identified as low and slow developing (Macrombe et al. 2014, Estep and Sanscrainte 2024), Parker et al. (2020) reported 48% of tested Florida populations being either resistant or developing resistance.

The unavailability of field mosquitoes prevented the comparisons between FPs and corresponding AIs. However, we were able to establish baseline laboratory resistance levels of differentially insecticide-pressured populations of *Ae. aegypti* and *Ae. albopictus* in SJC to two pyrethroids used in our IVM program. The results suggest implementing resistance management strategies and routine monitoring of target populations. The CDCBB would be the practical operational method for its feasibility, but it requires follow-up by other methods for confirmation. Further studies with adjuvant assays using PBO will help identify the capability of PBO to abolish the resistance acquired for AIs. As laboratory test results do not always correlate to the field (Vontasa et al. 2019, Richards et al. 2020), further observations with field studies and biochemical/molecular mechanism testing to determine which insecticide efficacy are required. This can provide important information for evidence-based decision-making in the control program and help improve mosquito control operations.

Furthermore, this study contributes further evidence for previous study results on the limited efficacy of PBO synergism in overcoming high resistance to pyrethroids in mosquitoes, including *Ae. aegypti* (Koou et al. 2014, Cornel et al. 2016, Marcombe et al. 2019, Riveron et al. 2019, Scott et al. 2021, Zuharah and Sufian 2021, Zhou et al. 2022). We detected the limited efficacy of PBO synergism in *Ae. albopictus* as well. PBO is believed to synergize the effects of pyrethroid insecticides by reducing the effects of the metabolic resistance mechanism, primarily reducing the detoxifying capabilities of monooxygenases (e.g., Cytochrome P450) (Zhou et al. 2022). As suggested by the present study the main resistance mechanism of *Ae. aegypti* in SJC would be *kdr*, and therefore, restoring susceptibility by PBO should be limited. The main mechanism in the resistant *Ae. albopictus* population in SJC could either not be elevated monooxygenase or the maximum carrying capacity of PBO (Zhou et al. 2022) to act upon the enzyme has been exceeded.

In conclusion, the study demonstrated widespread pyrethroid resistance in *Ae. aegypti* in SJC. *Ae. albopictus* was detected as resistant to Aqualuer 20-20, only in the

highest insecticide-pressured zone, indicating the low resistance development rates. PBO synergized insecticides have limited efficacy against highly pyrethroid-resistant populations of *Ae. aegypti* and *Ae. albopictus* in SJC. The results provide insights for transitioning to alternative insecticides or considering alternative techniques such as the sterile insect technique. Understanding the underlying mechanism conferring resistance in a population is important in selecting synergized formulations.

The study highlights the need for routine monitoring and updating of the resistance status/levels in target populations for formulated products used in mosquito control programs. Being more user-friendly and less expensive, the CDCBB would be the routine testing method to detect the development of resistance, which is followed up with more robust methods for confirmation and best-guided subsequent remedial measures.

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Scientific Note

COMPARING HUMAN LANDING RATE COUNTS WITH BG COUNTER 2 COLLECTIONS IN THE FLORIDA KEYS

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ABSTRACT

The human landing rate count (HLRC) is an important technique to measure mosquito biting pressure and evaluate key mosquito control district thresholds, but suffers from inconsistencies and other disadvantages that could be improved by use of an automated collection system. We compared mosquito collections from 15-min HLRC with those from a BG Counter 2, an autonomous mosquito collection system that counts mosquitoes and wirelessly transmits data to an end user. The experiment was conducted at two different locations in the Florida Keys in both late and early rainy seasons. Results showed variability in collections between HLRC and BG Counters in part due to known shortfalls of the HLRC method, indicating that the automated system could replace HLRC at some locations and should be evaluated further.

Key words: *Aedes taeniorhynchus*, BG Counter, Florida Keys, Human Landing Rate Counts, Surveillance

The human landing rate count (HLRC) is an important technique to measure mosquito biting pressure and, therefore, evaluate key mosquito control district thresholds, such as the decision to apply space sprays of adulticides (Hribar et al. 2022). However, there are drawbacks to this technique. For example, it cannot be guaranteed that the same mosquito control district inspector will always take the HLRC, and mosquitoes may be more or less attracted to different people (Ellwanger et al. 2021). Also, scheduling conflicts may lead to variation in timing of HLRCs from day to day, leading to variation in mosquito communities present for sampling (O'Meara 1976). These issues could be remedied by replacing HLRC with automated mosquito surveillance traps programmed to collect mosquitoes for a set period at a specific time of day. Florida Keys Mosquito Control District (FKMCD) inspectors perform 1-min HLRC at several locations every weekday between 7:00 a.m. and 9:00 a.m. to estimate density and inform treatment thresholds of the black salt marsh mosquito, *Aedes taeniorhynchus* Weidemann. We designed an experiment to compare HLRC of *Ae. taeniorhynchus* with counts from an automated trap system – the BG Counter 2 (Biogents, Regensburg, Germany) – at two locations in the Florida Keys with the objective to determine whether HLRC could be replaced by the system. The FKMCD has been experimenting with the BG Counter system since 2015 (Pruszynski 2016) and aims to incorporate them into daily operations by replacing HLRC [1]. If the BG Counter 2 system could provide equivalent data to HLRC, the efforts of FKMCD inspectors could be redirected to other high-value activities, for example, to search for larval habitat in mangrove swamps and hardwood hammocks within the District.

The BG Counter 2 is a programmable mosquito surveillance device that can differentiate mosquitoes from non-mosquito insects by size when passed through an infrared barrier (Biogents 2020). This information is then wirelessly transmitted in 15-min intervals to a webpage managed by the user. The BG Counter system used in this study consisted of a BG Counter 2 paired with a BG Pro trap (Biogents, Regensburg, Germany) which uses a solar-powered 12 V fan to pull host-seeking mosquitoes attracted by compressed CO_2 released in 4 s bursts at 50 g/h into a net. The experiment took place over 11 consecutive days in late rainy season in August/September 2022 and 12 consecutive days in the early rainy season in June

2023 with sunrise occurring between 6:40 a.m. and 7:10 a.m., respectively. Two locations, Koehn and No Name, were chosen as study sites because they were sites where HLRC were collected regularly, BG Counter systems were already in place, and high biting pressure populations of *Ae. taeniorhynchus* were expected. Koehn is located on Big Pine Key, FL (24.712777 N, -81.373056 W), and No Name is located on No Name Key, FL (24.700278 N, -81.328333), and the two sites are separated by approximately 5 km. Since the shortest duration for collection data for the BG Counter 2 is 15 min, the experiment was designed to compare 15 min BG Counter system collections to 15 min HLRC collections instead of the standard 1 min HLRC collections used operationally by FKMCD. Both BG Counter system and HLRC collections were conducted in the mornings: Koehn collections took place 6:45 – 7:00 a.m. and No Name 7:15 – 7:30 a.m. Inspectors conducting HLRC worked in pairs, with one person serving as bait and the other collecting landing mosquitoes with a battery-powered hand aspirator (Clarke, St. Charles, IL); roles were reversed at the second location. HLRC locations at each study site were 15 m away from the BG Counter system to limit differences in species distribution recruitment (Brown et al. 2008). The BG Counter 2 was programmed to run simultaneously with the HLRC collections, and after the 15 min collection period, inspectors removed the collection nets from the BG Pro Traps. Mosquitoes from both HLRC and BG Counters were stored in a -80 °C freezer for later counting and identification to species in the laboratory. A chi-square test of independence was used to determine if there was a significant difference in *Ae. taeniorhynchus* collections between the BG Counter system and HLRC methods. A Wilcoxon-Signed-Rank Test was used to investigate significant differences between HLRC and BG Counter system counts from each location during early and late rainy seasons and was paired with a Kendall Tau's correlation coefficient to determine the strength of the linear relationship ($\alpha = 0.05$). Each BG Pro Trap mosquito collection was tallied manually to verify automated counts performed by the BG Counter 2.

A total of 2,274 mosquitoes (97% *Ae. taeniorhynchus*) were collected at the Koehn site, and 858 mosquitoes (99.7% *Ae. taeniorhynchus*) were collected at the No Name site. The remaining 3% and 0.3% of collections, respectively, consisted of *Aedes condolescens* (Dyar & Knab), *Aedes tortillis* (Theobald), *Culex erraticus* (Dyar & Knab), *Culex bahamensis* (Dyar & Knab), *Psorophora johnstonii* (Grabham), and *Deinocerites cancer* (Theobald) and were excluded from further analysis. There was no significant difference in species composition between the BG Counter and HLRC methods at either site (X^2=2.11, P=0.14 at Koehn and X^2=2.75, P=0.97 at No Name). Manual counts of BG Pro Trap mosquito collections indicated that the BG Counter 2 produced an overall accuracy of 73% (±21%) at Koehn and 86% (±28%) at No Name. Mosquito abundances were notably higher during the early rainy season of 2023, with 1,976 more mosquitoes collected at Koehn and 810 more at No Name compared to those of the late rainy season of 2022 (Fig. 1). The Wilcoxon-Signed Rank test indicated no significant differences between the BG Counter 2 and HLRC methods at the Koehn site during the late rainy season 2022 ($W = 16.0$, $p = 0.238$), with Kendall's tau indicating a weak, non-significant positive correlation ($\tau = 0.23$, $p = 0.34$). In contrast, the early rainy season of 2023 at Koehn showed a significant difference between the two collection methods ($W = 6.5$, $p = 0.018$), though the correlation remained weak and non-significant ($\tau = 0.05$, $p = 0.84$). At the No Name site, the late rainy season 2022 data also showed no significant difference ($W = 9.5$, $p = 0.117$) but with a weak, non-significant negative correlation ($\tau = -0.12$, $p = 0.68$). However, during the early rainy season of 2023 at No Name, there was a significant difference between collection methods ($W = 2.0$, $p = 0.0015$) accompanied by a moderate and statistically significant positive correlation ($\tau = 0.46$, $p = 0.039$).

This experiment, designed to determine whether an automated mosquito collection system could replace the HLRC at two sites in the FKMCD, revealed various considerations for evaluating this change. First, the BG Counter system collected more mosquitoes than the HLRC at the Koehn study site but fewer mosquitoes than HLRC at the No Name site. Variation in human odors resulting from rotation of the two inspectors conducting the HLRC between the two sites could have impacted HLRC collection numbers, whereas the regulated CO_2 produced by the BG Counter system would have been less likely to impact collections. These data could be interpreted as an advantage of the BG Counter system to provide a uniform, comparable estimate of biting pressure across two or more sites. Depending on the threshold established at a mosquito control district that would trigger control activities, differences between BG Counter system and HLRC collection data could be irrelevant – in particular, compared to the likely higher uniformity and reliability of data from the automated system. One potential drawback for some mosquito control districts is that the BG Counter 2 is not yet able to identify collections to species. However, this is not a disadvantage for these and other sites in the wetlands where *Ae. taeniorhynchus* is the predominant species in all collections and the principal target of control.

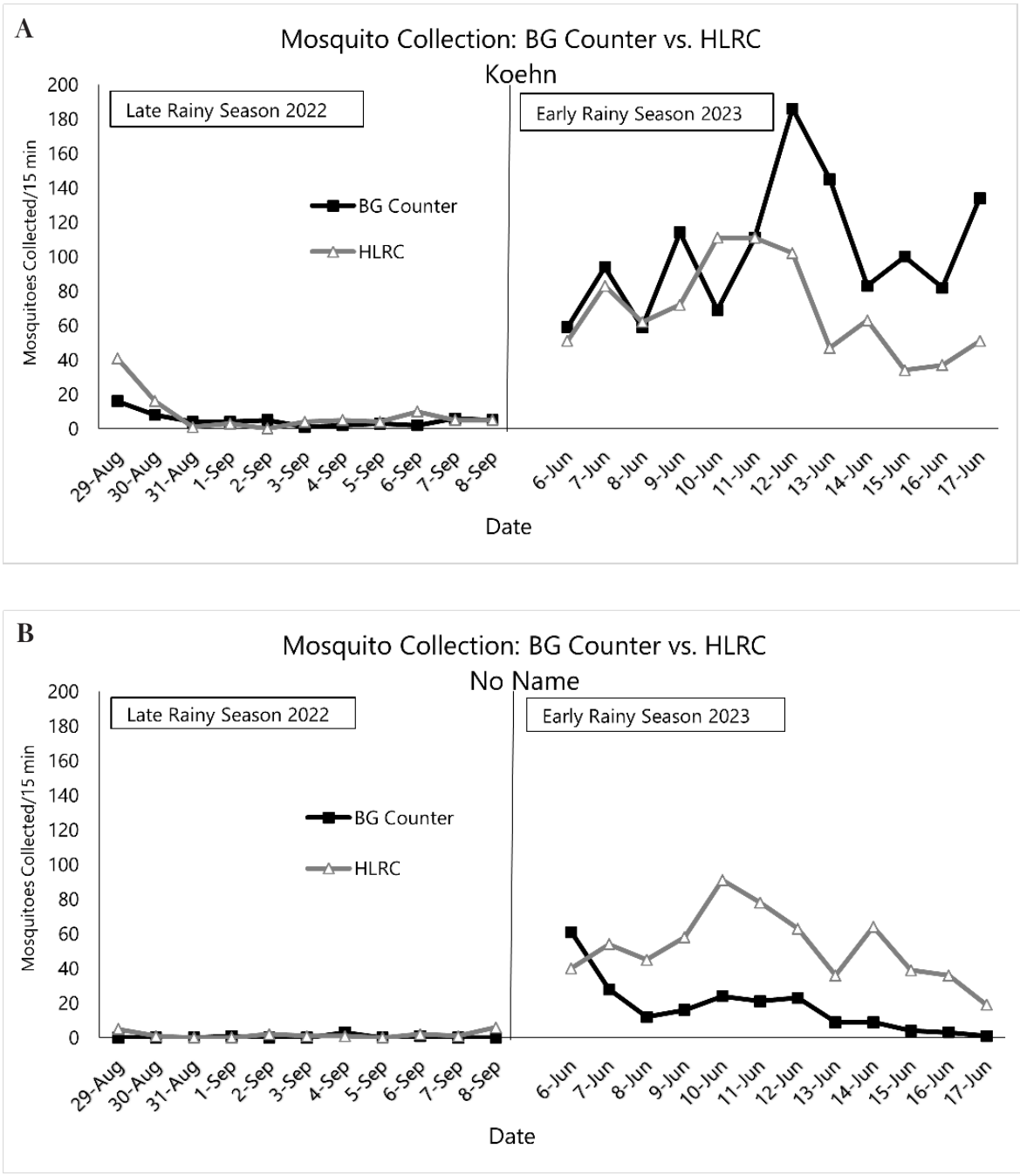

Figure 1. BG Counter 2 and human landing rate counts (HLRC) at the Koehn **(A)** and No Name **(B)** sites from 15 min collection periods across two seasons – the late rainy season 2022 (plots to the left of the vertical black line) and the early rainy season 2023 (plots to the right of the vertical black line).

Accuracy of the BG Counter has been investigated previously [6,7], indicating higher accuracy associated with higher density of mosquitoes. However, our data indicate lower accuracy (i.e., 73% at the Koehn site) associated with higher density of mosquitoes. One explanation for apparent poor performance of the BG Counter 2 is that mosquitoes that had already been counted by the automated system were observed escaping from the BG Pro Trap net while removing it from the device. This shortfall of our method to measure the accuracy of the BG Counter 2 suggests further evaluation is needed to establish the accuracy of the BG Counter 2. Similarly, mosquitoes were also observed flying out of the hand-held aspirator collection tube during HLRC collections due to low vacuum pressure from overcrowding. Multiple tubes had to be used during the 15-minute HLRC collection periods. The relative impact of experimental design on the accuracy of the two collection methods is not possible to determine from our data, therefore, additional trials may be needed to inform the decision to rely on one or the other method for detecting FKMCD control thresholds. Lacking additional data, our findings indicate that HLRC and BG Counter system methods could both be essential for informing operations of mosquito density and contribute, along with service requests and CDC trap data as important indicators for District control activities [1].

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INVESTIGATION OF INSECTICIDE RESISTANCE PROFILE OF ST. JOHNS COUNTY POPULATIONS OF *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* TO PERMETHRIN

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ABSTRACT

The prolonged use of permethrin-based products over 20 years has posed a risk of developing insecticide resistance in mosquitoes in St. Johns County, Florida, where *Ae. aegypti* and *Ae. albopictus* co-exist. Insecticide resistance to permethrin was investigated on the two invasive *Aedes* mosquitoes collected from St. Augustine, St. Johns County. Centers for Disease Control and Prevention (CDC) bottle bioassays on individual *Aedes* mosquitoes were conducted for 2 hours. At the diagnostic time, the mortality rates of *Ae. aegypti* and *Ae. albopictus* were 6.59% and 55.36%, respectively. This suggests that both *Aedes* species are resistant to permethrin. Based on a log-rank test, their mortality rates were significantly different ($p < 0.001$). The finding indicates the inefficiency of permethrin-based products targeting St. Augustine *Ae. aegypti* and *Ae. albopictus* populations.

Key words: mosquito control, invasive *Aedes*, permethrin, pyrethroid

INTRODUCTION

Two invasive mosquito species found in Florida, *Aedes aegypti* and *Aedes albopictus*, have been identified as vectors of multiple arboviruses, including dengue, chikungunya, and Zika, in the region (Richards et al. 2012, McCarthy 2016; Coatsworth et al. 2022). *Aedes albopictus* was first found in Florida in 1986 and then spread to St. Johns County in 1989 (O'Meara et al. 1995). *Aedes aegypti* had been present in St. Johns County before the invasion of *Ae. albopictus*, but it was displaced by *Ae. albopictus* in the early 1990s in the region (Dixon et al. 2020). However, after approximately 25 years, *Ae. aegypti* began to reemerge in St. Johns County in 2016 (Xue et al. 2020). Now, the two *Aedes* species are both prevalent in St. Augustine, St. Johns County (Aryaprema et al. 2024).

Anastasia Mosquito Control District (AMCD) has intensified its surveillance and control efforts targeting adult mosquitoes. These efforts included ground ultra-low volume (ULV) spraying with permethrin-based products, residential property barrier spraying with bifenthrin, and thermal fogging of vegetation areas using Duet® (active ingredients-1% Prallethrin, 5% Sumithrin, and 5% piperonyl butoxide, Clarke, Illinois). However, the effectiveness of ULV spraying with permethrin-based products on mosquito control has been gradually declining, likely because AMCD has used permethrin-based products for over 20 years in this region. To pinpoint the cause of this issue, AMCD and their collaborators have conducted various insecticide resistance tests, targeting *Aedes* mosquitoes against permethrin-based products (Wait et al. 2017, Aryaprema 2021, Sanchez-Arroyo et al. 2022, Wang et al. 2023, Kuppe et al. 2024), suggesting the development of insecticide resistance to permethrin. However, most of these studies were conducted using direct topical application, larval bioassay, and semi-field trials, rather than the widely employed CDC bottle bioassay, making it challenging to compare the results from other studies. The purpose of this study is to quantify and compare the resistance levels of *Ae. aegypti* and *Ae. albopictus* to permethrin in this region using the CDC bottle bioassays so that resistance level could be integrated for regional or national-level studies.

Eggs of *Aedes* mosquitoes were collected using ovitraps throughout St. Augustine, St. Johns County, during the summer of 2024 (Figure 1). The collected

Figure 1. Locations of collecting sites of *Ae. aegypti* and *Ae. albopictus* in St. Augustine, St. Johns County, Florida. Collecting sites are marked with red circles. This map was generated using QGIS 3.34.15 (QGIS Development Team 2023).

eggs were delivered to AMCD for rearing. The rearing condition at AMCD insectaries was 26.5 ± 1 °C, 80 ± 10% relative humidity, and a 14h light: 10h dark photoperiod. Individual-level CDC bottle bioassays were conducted on the reared *Ae. aegypti* and *Ae. albopictus* that were 3 to 5 days old to assess their resistance to permethrin. The modified CDC bottle bioassay to assess insecticide resistance in individuals would facilitate future analysis to identify individual genotypic insecticide resistance and subsequently analyze the relationship between specific genotypes and phenotypes. Test bottles were coated with 1 ml of the permethrin diagnostic dose (43 µg/ml) for treatment, and one control bottle was coated with 1 ml of acetone. We introduced one *Aedes* mosquito into each test bottle, whereas 15-20 mosquitoes were introduced into a control bottle. The maximum number of test bottles was set as 30 for one experiment round. We followed the instructions of McAllister and Scott (2020) for other experimental conditions for CDC bottle bioassay. Knockdown was recorded at 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes. Individuals exhibiting erratic behavior or lying in a rigid state were considered dead. A log-rank test was conducted to compare the survival rate of *Ae. aegypti* and *Ae. albopictus* using Python 3.10 with the lifelines library (Davidson-Pilon 2019), which is a commonly used method for comparing survival rates over time. Since some individuals remained alive at the end of the two-hour experiment, we analyzed the difference in survival rates between the two species.

A total of 91 *Ae. aegypti* and 56 *Ae. albopictus* were tested. Their mortality rates over time are shown in Table 1. At the diagnostic time, 10 minutes after the introduction into the test bottle, the morality rates of *Ae. aegypti* and *Ae. albopictus* were 6.59%, and 55.36%, respectively. Even at the end of the experiment, 29 *Ae. aegypti* (31.87%) and one *Ae. albopictus* (1.79%) were not knocked down. According to the CDC manual (McAllister and Scott 2020), resistance to permethrin for these two species is determined when the mortality rate is less than 90% at 10 minutes. Therefore, we identified *Ae. aegypti* and *Ae. albopictus* populations as resistant. In addition, the log-rank test indicates the

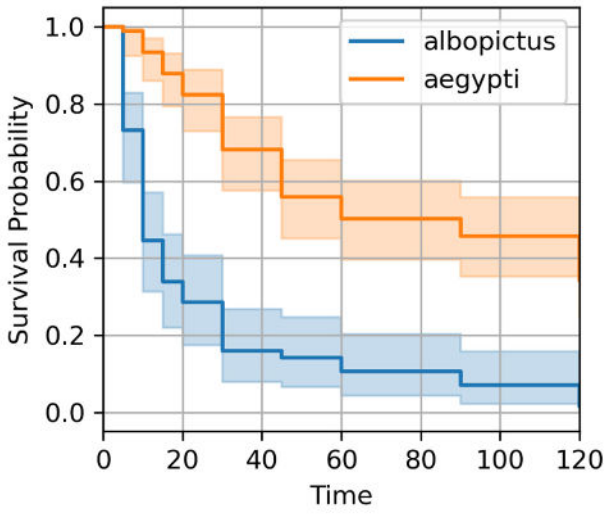

Figure 2. Kaplan-Meier survival curves of survival *Ae. aegypti* and *Ae. albopictus*.

survival rates of *Ae. aegypti* and *Ae. albopictus* from the St. Augustine were significantly different ($p < 0.001$) (Figure 2).

We found that both *Ae. aegypti* and *Ae. albopictus* collected from St. Augustine are resistant to permethrin. Even though this study was conducted with different experimental methods from previous studies, making

Table 1. Mortality rates (%) of *Aedes aegypti* and *Aedes albopictus* under exposure to permethrin for 120 minutes. Diagnostic times are marked with bold.

Time (min)	0	5	**10**	15	20	30	45	60	90	120
Ae. aegypti	0.00	1.10	**6.59**	12.09	18.68	32.97	45.05	50.55	56.04	68.13
Ae. albopictus	0.00	25.00	**55.36**	66.07	71.43	83.93	85.71	89.29	92.86	98.21

the quantitative comparison difficult, our findings are consistent with previous studies reporting insecticide resistance of *Ae. aegypti* and *Ae. albopictus* from St. Johns County (Waits et al. 2017, Wang et al. 2023). Through this study, we provided results that can be comparable with other populations. Further intensity or mechanism testing with piperonyl butoxide is required to determine the strength of the resistance and the mechanism involved in developing the resistance. This information highlights the inefficiency of permethrin-based products against *Ae. aegypti* and *Ae. albopictus* populations from St. Augustine and underscores the need to use alternative insecticides for mosquito control.

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SUBMITTED ABSTRACTS OF THE 96TH ANNUAL FLORIDA MOSQUITO CONTROL ASSOCIATION'S MEETING

Rose Center, Orlando, FL, November 4-7, 2024

Subject Editor: Rui-De Xue

Assessing field populations of *Aedes taeniorhynchus* susceptibility to spinosad: insights into larval density and water quality effects

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Indian River Mosquito Control District, Vero Beach, FL

The aerial application of larvicides is a vital method for managing salt marsh mosquitoes, especially *Aedes taeniorhynchus*, within the Indian River Mosquito Control District. Despite its importance, there have been instances where spinosad (Censor®) treatments did not effectively control *Ae. taeniorhynchus*. To investigate the cause of these control failures, we conducted laboratory bioassays to assess the local *Ae. taeniorhynchus* population's resistance to spinosad, as well as the impact of application rates, larval density, organic content/salinity of field water on efficacy. Additionally, field bucket catches were performed to verify the aerial application rates.

Laboratory bioassays confirmed that the local *Ae. taeniorhynchus* populations are susceptible to spinosad, similar to the USDA susceptible colony. The bioassay results also showed that Spinosad achieved nearly 100% mortality at our standard application rate of 12 lbs./acre (equivalent to 0.5 ppm 12 lbs./acre for a 6-inch water depth) across three larval densities (25, 50, and 100 larvae/cup). Even at a lower concentration of 0.25 ppm (equivalent to 6 lbs./acre for a 6-inch water depth), spinosad still resulted in close to 90% mortality at all three larval densities. The bioassays also indicated no significant difference in larval mortality between field water (with high organic content and salinity) and 10 ppt tap water at various spinosad concentrations with 25 larvae per cup; increased mortality was noted at 50 larvae per cup with a concentration of 0.05 ppm and at 100 larvae per cup with concentrations of 0.1 ppm and 0.05 ppm.

Results from field bucket catch collections revealed that the aerial distribution of Censor was inconsistent, with application rates frequently falling below 3 lbs./acre (equivalent to 0.125 ppm). This irregular distribution likely contributes to the observed control failures in the field. In conclusion, ensuring consistent and adequate distribution of Censor is essential for the effective control of *Ae. taeniorhynchus* through aerial treatments.

Preliminary evaluation of toxicity of two essential oils to *Aedes taeniorhynchus* larvae.

Lawrence J. Hribar
Florida Keys Mosquito Control District, Key West, FL

The potential of two essential oils, Frankincense oil (*Boswellia* spp.) and Myrrh oil (*Commiphora* spp.) to kill larvae of the Black Salt marsh Mosquito, *Aedes taeniorhynchus* (Wiedemann) was investigated. A stock solution of 0.1% Tween® 20 was prepared and used as a diluent for all tests. Tween® 20 is an emulsifying agent used to prepare stable oil-in-water emulsions. A range of dilutions (0.5% to 0.001%) was prepared for both oils. Distilled water and the stock Tween® 20 solution were used as negative controls; a 0.1% solution of naled was used as a positive control. Over 95% of larvae tested died when exposed to the lowest concentration of Frankincense oil whereas less than half died at the same concentration of Myrrh oil. There was zero mortality in the distilled water and Tween® 20 negative controls and 100% mortality in the naled positive control. Based on these limited trials, Frankincense oil appears to be more toxic to *Aedes taeniorhynchus* larvae than Myrrh oil.

Effects of mosquito larvicides and adulticides applied via truck mounted and aerial spray on honey bees (Apis mellifers) in Florida

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The use of Ultra Low Volume (ULV) mosquito adulticides is common in Florida, particularly in urban areas, as they are highly effective at controlling mosquitos. Additionally, there are many Florida mosquito control districts in which water bodies are treated with mosquito larvicides. The impact of some of these chemicals on honey bees has been studied, but not as much is known about how simultaneous exposure to these chemicals in realistic field scenarios will impact overall honey bee colony strength. We hypothesize that residues of mosquito control products used in truck-based and aerial applications will be found inside honey bee hives, though at levels low enough not to cause acute toxic responses. In this study, we determined the impact of field realistic mosquito control practices on colony strength parameters by placing 15 new honey bee colonies in three "hotspot" treatment sites (five colonies per site) in which mosquito control operators treat three to five times each site. We also placed 15 new colonies in three sites (five colonies her site) receiving little to no mosquito treatment applications (negative control sites). The "hotspot" treatment sites were treated with Mosquito Mist II®, Naled®, and Vectobac12AS®. Colony strength parameters were measured before and after the three-month treatment period and samples of hives matrices (pollen, nectar, bees, brood) were collected from all colonies and analyzed to determine the residue levels of mosquito control product present in the hives. Some mosquito control product residues were found at a low concentration in hive matrices (0.06 – 70.99 ng/g). No significant differences were observed in colony mortality and health parameters between colonies located in treated and control sites. The resulting data will be used to inform best management practices for mosquito control programs and apiculture in the future.

Mapping our way to mosquito control success: Our Journey With ArcGIS Online

Robert Cartner
Beaufort County Mosquito Control, Beaufort, SC

Finding solutions to effectively collect and maintain data is critical for a Mosquito Control program. While having the ability to properly document data is key, providing a cost-effective solution is impactful as well. With the assistance of Beaufort County's GIS Department, Beaufort County Mosquito Control has developed a database built completely on ArcGIS Online (Esri, Redlands, CA). Technicians document field data on a tablet connected to a cellular network and supervisors can view the data in real-time on maps with pre-configured analysis parameters. This streamlined workflow allows control activities to be scheduled more quickly. By utilizing software already in operation by the county government and County GIS analysts, Mosquito Control is able to save money and operate more efficiently. Mosquito Control will continue to develop the database system and incorporate new GIS techniques as they become available.

Semi-field evaluations of ground and aerial ULV applications using ReMoa Tri against deltamethrin-resistant *Aedes aegypti* from Collier County, Florida.

Decyo McDuffie, Sara Kacinskas, Casey Parker-Crockett, Leanne Lake, Samantha Ramirez-Lachmann, Haley Johnson, Rachel Bales, Keira J. Lucas
Collier Mosquito Control District, Naples, FL

New intervention methods and product formulations are needed to better control pyrethroid-resistant *Aedes aegypti* populations and mitigate the risk of mosquito-borne disease. ReMoa Tri is a novel adulticidal space spray that utilizes a different mode of action than the commonly used adulticides, pyrethroids and organophosphates. As a triple-action space spray, ReMoa Tri combines 3 components: Fenpropathrin, a mixed-type I/II pyrethroid; abamectin, a macrocyclic lactone; and C8910, a patented fatty acid chain. Prior studies performed by Collier Mosquito Control District showed that ReMoa Tri is effective at treating type I pyrethroid-resistant *Ae. aegypti* mosquitoes. To further validate these results and the performance of ReMoa Tri, we conducted a semi-field evaluation using ground and aerial ULV applications with field-caught deltamethrin-resistant *Ae. aegypti* and a susceptible *Ae. aegypti* laboratory strain. Ground evaluations tested ReMoa Tri and a type II pyrethroid, Deltagard. While ReMoa Tri was equally effective against Collier's deltamethrin-resistant *Ae.*

aegypti and the susceptible laboratory strain, Deltagard was ineffective against Collier's resistant strain. Similarly, aerial evaluations also showed that ReMoa Tri was equally effective against Collier's deltamethrin-resistant *Ae. aegypti* strain and susceptible laboratory strain. This study further confirms ReMoa Tri's potential as an effective alternative to pyrethroid-based adulticides, both in ground and aerial applications, for managing pyrethroid-resistant *Ae. aegypti.*

Resistance Status of *Culex quinquefasciatus* in St. Johns County, FL
Connor Kuppe, Olivia Sypes, Vindhya Aryaprema, Kai Blore, Muhammad Farooq, Steve Peper
University of Florida, Department of Entomology and Nematology and Anastasia Mosquito Control District

Insecticide resistance is a primary concern of integrated mosquito management programs as resistance can often lead to control efficacy loss. As such, the resistance status of *Culex quinquefasciatus* is of heightened concern to Anastasia Mosquito Control District due to the vector status and prevalence of the species within St. Johns County. Here, we examine the resistance profile of the species through topical application assays, CDC bottle bioassays, molecular assays, and control efficacy testing. Topical application results indicated a resistance ratio at LD_{50} of 14.49 against permethrin. CDC bottle bioassay results showed heavy resistance against multiple active ingredients regularly used within Anastasia Mosquito Control Districts control program, including, permethrin, sumithrin, chlorpyrifos, and naled. Enzyme inhibitor use in conjunction with CDC bottle bioassays demonstrated strong metabolic resistance through increased oxidase and esterase activity. Current limitations within this study in regard to knock-down resistance genotyping bound thorough analysis of the population's resistance towards pyrethroids through *kdr*, however a preliminary finding of a high abundance of the 1014F allele does provide validation to the high prevalence of phenotypic *kdr* observed within the bottle bioassays. Finally, wind tunnel analysis indicated no reduction in control efficacy of the population against Aqua Kontrol 30-30, as 100% mortality was achieved at low, medium, and high concentration rates formulated as per label instructions. Results provide the foundation for recurrent resistance monitoring and can offer insight into management strategies for these two important disease vectors.

Brevard County Mosquito Control District's evolving integrated mosquito management strategies
Jonathan Linder, Joseph Faella, Maxwell Reynolds, Bridget Coffey-Picco, Jonathan Koagel
Brevard County Mosquito Control District, Titusville, FL

An overview of Brevard County Mosquito Control District's Integrated Mosquito Management (IMM) strategies and how changes to adulticide and larvicide delivery, equipment innovations, pilot impoundment projects, and process improvements have appeared to affect mosquito populations and pesticide usage over time.

Incorporating new equipment into Brevard County Mosquito Control District's surveillance program
Maxwell Reynolds, Bridget Coffey-Picco, Jonathan Linder, Chris Murphy, Jacyln Wertz, David Smith, Joseph Faella
Brevard County Mosquito Control District, Titusville, FL

A summary of Brevard County Mosquito Control District's current surveillance trapping regimen, new trapping equipment purchased with the CDC CK-19-1904, Epidemiology and Laboratory Capacity for Prevention and Control of Emerging and Infectious Diseases (ELC) grant awarded through FDACS, and the new equipment's future uses.

Leveraging biological control for pre-season impoundment mosquito population reduction
Bridget Coffey-Picco, Maxwell Reynolds, Jonathan Linder, Chris Murphy, Thomas Tucker, Jamaine McWhite, Richard Briggs, Kevin Blaylock, Joseph Faella
Brevard County Mosquito Control District, Titusville, FL

In the 1950s and 1960s, over 30,000 acres of shallow salt marshes in Brevard County, Florida, were converted into mosquito control impoundments to mitigate salt marsh mosquito species *Aedes sollicitans* and *Aedes taeniorhynchus* populations. These mosquitoes oviposit on exposed wet sediment, hatching eggs upon inundation by tides or rainfall. Traditional mosquito control methods in these impoundments focus on source reduction, eliminating breeding sites for egg-laying females, by closing water control structures and filling the impoundments with pumps, and applying

larvicides to control the first brood prior to emergence. Brevard County Mosquito Control District (BCMCD) tested an alternative approach to reduce reliance on biorational larvicides. This approach involved daily monitoring of a 40-acre impoundment using CDC light traps baited with dry ice and site inspections to identify breeding hotspots. Instead of using larvicide, the water level was temporarily increased, allowing natural fish populations to prey on mosquito larvae in shallow dense grassy areas that fish cannot normally access. This method was further scaled to a 1,700-acre impoundment by augmenting the impoundment's pump with a portable pump to rapidly elevate water levels and introducing 16,000 native *Gambusia holbrooki* (mosquitofish) to assist with larval control. A weekly CDC trap surveillance comparison over several years showed a significant (80%) reduction in the initial mosquito emergence from the impoundment. Additionally, larvicide usage was reduced by 45% compared to the average of the previous four seasons. These results demonstrate the potential for integrated, environmentally friendly mosquito control strategies that reduce pesticide usage and consequently assist in managing local salt marsh mosquito resistance while maintaining effective control of mosquito populations through briefly increasing impoundment water elevation by 8 to 10 inches above historic target water elevations.

Activation of Anastasia Mosquito Control District's aerial emergency contract following a non-disaster event
Whitney A. Qualls
Anastasia Mosquito Control District, St. Augustine, FL

This presentation outlines the operational response of the Anastasia Mosquito Control District (AMCD) during a non-disaster event requiring aerial emergency interventions. AMCD manages a service area of 609 square miles in St. Johns County, Florida, including 115,000 acres of flood-prone woodlands and 60,000 acres of federally and state-protected lands. Between August 28 and September 17, 2024, a surge in mosquito populations, indicated by a 50% increase in trap counts, necessitated rapid activation of aerial spraying measures. Despite logistical challenges, including AMCD grounded aircraft and operational limitations, AMCD successfully coordinated resources to mitigate the public health risks associated with rising mosquito activity. The presentation highlights the integration of surveillance, strategic use of AMCD's emergency contract for aerial mosquito control, and the collaborative efforts of AMCD staff and external partners like Vector Disease Control International. We highlight the importance of adaptive planning and partnerships in managing vector populations effectively in dynamic environments.

Down the Crab Burrow: Studying the microbiome of *Deinocerites cancer*
Alexandra Bauer, Daniel Pérez-Ramos, Lawrence Reeves, Eric Caragata
University of Florida/ Florida Medical Entomology Laboratory, Vero Beach, FL

Microbial communities play a critical role in influencing mosquito life cycles by impacting reproduction, development, immunity, and pathogen transmission. While larval habitat is a key factor in shaping the mosquito microbiota, the influence of the local environment on host-microbe dynamics remains understudied. This study investigates the highly specialized crabhole mosquito *Deinocerites cancer* to explore how larval environmental variability affects microbial diversity in both juvenile and adult mosquitoes.

We developed low-cost collection systems for *D. cancer* and conducted aseptic sampling of larvae and their developmental matrix (i.e., water and sediment) from 10 crab burrows during July and August 2024. Significant spatial variation in water physicochemical parameters, including iron, chemical oxygen demand, sulfide, nitrate, and conductivity, was observed. Burrows adjacent to ponds exhibited higher sulfide, nitrate, and conductivity compared to those near lagoon arms, implicating the water body as the main driver of physicochemical variation even on small spatial scales.

We expect the high variability of the larval environment to be reflected in the larval microbiome. Understanding how environmental conditions shape mosquito-associated microbial communities provides insight into key host-microbe-pathogen interactions, offering potential applications in vector control strategies.

Incriminating the vectors of deer malaria (*Plasmodium odocoilei*) at a Florida Deer Farm
Morgan Rockwell, Nathan Burkett-Cadena, Samatha Wisely, and Derrick Mathias
University of Florida/Florida Medical Entomology Laboratory, Vero Beach, FL

Deer malaria is a disease caused by *Plasmodium odocoilei*, the only *Plasmodium* in North America in white-tailed deer (WTD). To incriminate the vector, the mosquito feeding preference of WTD and the infection rate of *P. odocoilei* were determined. At a deer farm in Gadsden County, FL, mosquito species were collected using CDC light traps, a large-diameter aspirator, and pop-up resting shelters. Host use of mosquito and infection rate of *P. odocoilei* was determined using PCR and Sanger sequencing. It was found that *An. quadrimaculatus s.l.* (88.9%), *An. punctipennis* (83.3%), *An. crucians s.l.* (80.0%), and *Cx. erraticus* (80.0%) preferred to feed on WTD. The highest infection rate of *P. odocoilei* was found in *An. quadrimaculatus s.l.* (9.7%), followed by *An. punctipennis* (4.1%), and *An. crucians s.l.* (0.91%). This study determined that *An. quadrimaculatus s.l.* is the probable vector for *P. odocoilei* attributed to its high feeding on WTD and a high infection rate of *P. odocoilei*. Other potential vectors include *An. punctipennis* and *An. crucians s.l.*, because of their *P. odocoilei* infection and feeding preference of WTD. To fully incriminate *An. quadrimaculatus s.l.*, *An. punctipennis*, and/or *An. crucians s.l.* as the vector for *P. odocoilei* vector competence needs to be determined.

Creating accessible science: BEACONS online dashboard for invasive mosquito species
Olivia *R. Magaletta, Yesenia Sánchez, Bryan V. Giordano, Yoosook Lee, Lindsay P. Campbell*
University of Florida/Florida Medical Entomology Laboratory, Vero Beach, FL

In the southeastern United States, several invasive mosquito species of medical and veterinary importance pose a risk of arbovirus transmission to humans and livestock. To aid in surveillance efforts, an online dashboard has been developed that allows data sharing over broad regions and promotes coordinated monitoring across districts for the purpose of enhancing detection of emerging public health threats. The dashboard compiles and maps species occurrence records from data aggregators (i.e. GBIF), repositories, scientific literature, and provides a form for user submitted records hosted on the ArcOnline platform through the University of Florida. Prioritizing contributions of records for invasive mosquito species provides a manageable balance between data submission and sustained maintenance for tracking and communicating distributions for targeted mitigation strategies. This dashboard is freely accessible through the Mosquito BEACONS website. The dashboard currently contains over 225 thousand occurrence data points for 19 mosquito species from 1900-2024. Integrating digital dashboard mapping with analytical tools and real-time surveillance data will enhance communication on the spread of invasive mosquito species. The platform is positioned to expand its scope to effectively meet the needs of collaborators in mosquito control and public health and to serve as a resource for scientists and community members.

What lives inside a mosquito? Delving deep into the mosquito microbiota
Daniel W. Pérez Ramos, Marina M. Ramos, Kyle C. Payne, Bryan V. Giordano, Eric P. Caragata
University of Florida/Florida Medical Entomology Laboratory, Vero Beach, FL

Mosquitoes are naturally inhabited by diverse communities of microorganisms, which can alter their interactions with disease-causing pathogens or help to reduce their susceptibility to insecticides. As such, it can be useful for mosquito control programs to understand the impact of these microbes on their target mosquito populations. The mosquito microbiota varies because of extrinsic factors such as me, geography, seasonality, and between mosquito species. Further study is required in order to understand how these factors interact and shape the microbial communities of mosquitoes in Florida. For this reason, we profiled the microbiota of four mosquito species; *Aedes taeniorhynchus*, *Anopheles atropos*, *Anopheles crucians*, and *Culex nigripalpus*, collected from Vero Beach, Florida, during dry and wet seasons. Bacteria and fungi were profiled using 2x300bp 16s rRNA-based and ITS 1/ITS 2-based Illumina Mi-Seq, respectively. Florida mosquitoes had high bacterial richness and diversity, with community composition strongly influenced by both seasonality and host species. Mosquitoes were also infected by highly diverse fungi, but a single fungal isolate predominated across species and seasons. Our findings suggest that the environment can play an important role in shaping the mosquito microbial composition, but that some mosquito-microbe associations persist across environmental conditions.

An overview of Anastasia Mosquito Control District's pathogen surveillance program

Edward Zeszutko
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Anastasia Mosquito Control District, St. Augustine, FL

Arboviruses continue to be a public health concern on a global scale. Arboviral surveillance is an important aspect to any vector control or public health operation. The control of arboviral activity is most effectively accomplished through the management of vector species and the surveillance of viral activity. Anastasia Mosquito Control District utilizes sentinel chickens and mosquito pool testing for arboviral surveillance and is conducting an ongoing project to determine dog heartworm vector competency in multiple mosquito species. The results from these types of surveillance help guide abatement efforts for vector species and establish meaningful outreach materials.

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