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2020-2021 THE FMCA PRESIDENTIAL ADDRESS

James Clauson
Beach Mosquito Control District, Panama City, Florida

Good morning and welcome to the 93rd meeting of the Florida Mosquito Control Association. We are very happy to be meeting in person after a 2-year hiatus due to the Covid Virus. It is very nice to be at this wonderful venue, Hawks Cay Resort at Duck Key in the beautiful Florida Keys.

My name is James Clauson, and I am Director of Beach Mosquito Control District located in Panama City Beach. It has been an honor to serve as President this past year. This association is made up of many volunteers and I would like to recognize them for all the hard work they have put in. I would especially like to recognize our new Executive Director, Karen Crawford with CMC Associates. Karen has done a tremendous job of turning around our association, from a social club to a professional organization, that is financially very sound. My predecessor, Donnie Powers initiated this move, and I am proud that we are continuing in the right direction. Since we didn't have a meeting last year, I will share my Presidential Address time with Donnie.

I would first like to honor our Veterans. I would ask that if you are a Veteran of the Armed Forces; Navy, Army, Marines, Coast Guard or Air Force, please stand and be recognized. We thank all of you for your sacrifices. I hope we continue this recognition for future meetings.

Next, I would like to mention and highlight some of the other mosquito control and vector control organizations that I have been a member of throughout my career.

They are:

+ Mosquito and Vector Control Association of **California** (MVCAC)
+ Society of Vector Ecology (SOVE)
+ Northeastern Mosquito Control Association (NMCA)
+ Mid-Atlantic Mosquito Control Association (MAMCA)
+ **Georgia** Mosquito Control Association (GMCA)
+ **Louisiana** Mosquito Control Association (LMCA)
+ West Central Mosquito and Vector Control Association (WCMVCA)
+ Northwest Mosquito and Vector Control Association (NMVCA)
+ American Mosquito Control Association (AMCA)
+ International Forum for the Surveillance and Control of Mosquitoes and Vector-Borne Diseases (IFSCMVD), China

I think it is very important that professionals network and interact with others so we can all learn from each other. There is no sense in "re-inventing the wheel". I have picked up tips and BMPs (Best Management Practices) from other mosquito control districts who have given talks at other Mosquito Control Association meetings. I always learn something when I go to these meetings and I bring that back to my district.

Lastly, again welcome. I will turn over the balance of my time to Donnie Powers, President in 2019-2020.

Thank you and enjoy.

James Clauson

USING CHEMICALS TO CONTROL MOSQUITOES IN THE 21ST CENTURY: SOME OBSERVATIONS AND CHALLENGES IN THE U.S. AND AUSTRALIA

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Subject Editor: Rui-De Xue

ABSTRACT

This opinion paper was written at the editor's invitation to provide insights on the use of chemicals for mosquito control. Because this topic is of international significance, mosquito control workers from several locations in the U.S. and Australia are included as co-authors, including an industry representative. The categories addressed include geographical differences, public perceptions, toolbox challenges, regulations, resistance, exotic vectors/pathogens, along with looking to the future. This paper will benefit the readers by demonstrating how these topics of importance to mosquito control are viewed in several diverse geographic locations.

Key Words: Opinion paper, chemical control, larvicides, adulticides, public perceptions

INTRODUCTION

The need to apply chemicals to control mosquitoes can be divided into treatments for nuisance mosquito problems versus applications targeting mosquitoes potentially involved in disease transmission. The consideration for when, where and how to treat can vary greatly based on various factors with a major consideration being environmental protection.

One item that makes this group of co-authors particularly appropriate for this paper is the fact that they come from somewhat different backgrounds relative to their use of mosquito control chemicals. Most mosquito control programs strive to implement an Integrated Pest Management (IPM) technique with source reduction being the first choice where possible because it oftentimes is the most effective measure. When source reduction is not an option, larviciding and/or adulticiding typically remains an important part of this multi-disciplinary approach with all control actions dictated by surveillance information (Lloyd et al. 2018).

GEOGRAPHICAL DIFFERENCES WHEN IMPLEMENTING CHEMICAL CONTROL

It has interested us that the practice of applying mosquito larvicides and adulticides has evolved differently in various regions of the U.S. and elsewhere. For instance, while the use of both larvicides and adulticides is the norm in Florida (both for nuisance and disease-transmission situations), in many regions of California the use of adulticides is limited under both circumstances. In Queensland east-central Australia, where all mosquitoes are designated pests whether they are nuisance or disease vectors, larviciding is preferred with a minimal use of adulticiding.

SOME U.S. PERSPECTIVES. In both California and Florida, it is interesting to note an association that areas where agriculture is prevalent, overall the general public readily accepts the fact that using pesticides to control mosquitoes there is a necessary component of an IPM program (in our industry commonly known as Integrated Mosquito Management (IMM)). For much of the past century in Florida, citrus has been the primary agricultural crop where pesticide and herbicide use was widespread. However, over the past decade in Florida, citrus production has decreased dramatically due to the bacterial pathogen known as Citrus Greening (*Candidatus Liberibacter asciaticus*). The common use of chemicals originally intended for agricultural use, but applied for mosquito control purposes, which largely began after World War II, continues to this day. However, we commonly see that new people moving to the state (reportedly at the rate of approx. 900 individuals per day) are less tolerant of such pesticide use. In contrast, California has had regional differences in their acceptance of these practices for many years.

California mosquito control agencies pride themselves on using all the components of an IMM plan. However, when it comes to adulticiding, geographic differences become apparent. Wide area adulticiding is rarely used to address adult mosquitoes south of the Tehachapi Mountain range. However, in northern California (N. Cal), adulticiding is necessary to address mosquitoes infected with West Nile virus, along with the periodic need to combat "nuisance" mosquito problems. Much of this may be due to the lack of water and water-intensive agriculture in Southern California (S. Cal) as opposed to N. Cal. S. Cal, with its largely dense urban areas, has a Mediterranean-like climate, primarily with dry summers and moderate winter rainfall. Contrast this with N. Cal's Sacramento Valley, where over 500,000 acres of rice is grown each year which requires a consistent water level of approximately one foot from April to October, creating suitable habitats for *Culex tarsalis* and *Anopheles freeborni* (USDA 2020). North California is also home to large wildlife refuges that flood irrigate fields for waterfowl habitat *(*FWS 2021*)*. These intermittent floodings result in tremendous hatches of *Aedes* species, most notably *Ae. melanimon*, *Ae. vexans*, and *Ae. nigromaculus.*

Historically S. Cal mosquito control agencies have not had to deal with large numbers of mammal-biting mosquitoes. However this has recently changed with the introduction of *Ae. aegypti* and *Ae. albopictus.* Northern California agencies have always had to deal with mosquitoes resulting from farming practices and wildlife refuge management. While larviciding is the preferred method of control for all California agencies, the fiscal reality of larviciding vs. adulticiding these large acreages result in more adulticiding in N. Cal than in S. Cal.

The public in many of the N. Cal communities are accustomed to measures being taken for agricultural insect problems so this seems to translate to many N. Cal mosquito control agencies receiving a greater public acceptance of adulticiding compared to those in S. Cal and the San Francisco Bay area. Indeed in S. Cal in 2004, when WNV became a problem, wide-area adulticiding was limited. The reason given was that the public simply would not accept it. In contrast In N. Cal in 2005, while there was some backlash when adulticiding was initiated, the overwhelming response to the treatments was accepted as long as treatments only occurred when appropriate thresholds were met. While ideally politics should not play into science-driven responses, there does seem to be a link between conservative acceptance and liberal disapproval of the use of chemicals. While we are not aware of any surveys or studies supporting this observation, large areas of S. Cal and the San Francisco Bay area are commonly identified as liberal strongholds, while N. Cal is identified as being conservative. In comparison, unlike California, Florida does not seem to exhibit such clear geographical delineations concerning how mosquito control pesticides are perceived and used.

SOME AUSTRALIAN PERSPECTIVES. Mosquito control in Queensland is mandatory as dictated by legislation. A recurring theme discussed by our Australian authors, as they developed their contributions for this paper, was the strong opinion that without chemicals, there would be no effective mosquito control programs. If mosquito control chemicals were no longer available (*e.g.,* no longer allowed), it would be a serious setback in meeting their agencies' goals. It is believed that without chemical treatments "botanical gardens and parks would not be usable". *Bacillus thuringiensis israelensis* (*Bti*) is the primary larvicide with 20%-30% of the treatments using the insect growth regulator methoprene (applied either as sand granules or liquid).

Mosquito control must be efficient and cost-effective (Shepard et al. 2014), thus it must truly be providing a benefit. Cost-benefit analysis is an integral part of all decision-making including that for aerial and/or barrier treatments. When adult mosquito populations are targeted, it mainly involves barrier treatments to separate residents from sources of abundant adult mosquitoes (Lloyd et al. 2021; Mohd-Noor et al. 2021). Bifenthrin is the adulticide used for such barrier applications and care is taken in the treatment design to avoid any contamination of water bodies (as per label requirements). For example, one 2 km solid timber "wall" at the edge of a residential development is treated approximately 6 times a year which

protects thousands of nearby residents from mosquito problems. Other barrier treatments are used for treating vegetation in parks and at the interface between residents and salt marsh habitats (Qualls et al. 2012).

Adulticiding (commonly referred to as fogging in Australia) is rare but conducted when mosquito control staff experience "a population (of mosquitoes) rolling out of the swamp". Under these circumstances some ULV spray equipment is held in reserve for emergency response work. When fogging is implemented, the adulticide Twilight® (a phenothrin/piperonyl butoxide mix in a liquid hyrocarbon solvent) is used. In determining when and where adulticide applications are made, it is frequently stated that mosquito control is "about 90% scientific rigor and 10% artistic flair"!

PUBLIC PERCEPTIONS

Those of us who have been in the industry for several decades have seen how some of the public has held a simplistic impression that controlling mosquitoes is rather easy. The public perception is not always what we say but what the public "hears" making this work a challenging endeavor. Controlling mosquitoes is multi-faceted which includes understanding new regulations, the situation of a shrinking number of chemicals which are available, and striving to control a pest with an exceptional propensity to avoid or neutralize the toxin in a variety of ways.

SOME U.S. PERSPECTIVES. In most locations, the public believes mosquitoes should be controlled using an IPM approach but for many, this work should not include adulticiding. When a pathogen presents itself within a mosquito population that results in the need for adulticiding, the first public responses tend to be "Why are you not using IPM measures?" For example, Beyond Pesticides (a nonprofit organization which advocates against using pesticides) frequently suggests that same premise of thought. Much of this perception is due to the much less visible nature of larviciding, water management, and source reduction as opposed to adulticiding. The public simply does not notice the considerable efforts that occur before an adult mosquito intervention is needed. However, when they see the plume of an adulticide being released in their neighborhood by truck or aerially by an aircraft, they want to know why the other components of IPM were not employed.

There are a few reasons for this. One, it can be argued that overall, our industry has not done an effective job of public outreach regarding comprehensive mosquito control. It was not that long ago when a trained public outreach specialist in a mosquito control program was considered an expensive perk, as opposed to being a necessary component of an effective program. Having an expert in communicating the nuances of our programs is important in obtaining public acceptance of a fully integrated control program. Just describing the different species, their habitats, the regulations involved, etc. to the public can be a fulltime job that many agencies either do not have the resources to employ or simply choose not to do.

Second, adulticiding is seen as an invasion in the public space. Larviciding, water management and source reduction are generally conducted on a specific piece of property that members of the public feel they can avoid. When an adulticide application is made along the road or overhead, the individual's choice of avoiding it has been made much more difficult. They therefore may not be supportive of this method of control.

Along the central east-coast of Florida, several mosquito control programs contract aerial application of larvicides with a company which uses the fixed-wing Air Tractor 802A. Interestingly, while public acceptance of larviciding is sometimes greater than that of adulticidng, by some residents these aerial larviciding applications are perceived as being dangerous, especially because flight paths are commonly near (and frequently over) residential areas adjacent to salt marshes needing larvicide treatment. Even though the ability to make these flights is approved by the Federal Aviation Administration through a Congested Area Flight Plan, this still does not appease some residents' concerns about this practice which is necessary to adequately control these mosquitoes produced close to their property which are capable of flying long distances.

SOME AUSTRALIAN PERSPECTIVES. Public perception, attitudes and expectations were discussed amongst the 3 Australian co-authors recognizing that the use of specific terms is important for communicating with the public (Morse et al. 2019). For example, at one of the programs, chemicals are generally referred to as "products". In other locations, the term "products" refers to larvicides whereas adulticides are referred to as "chemicals". The reasoning for using the term "products" for larvicides is based on its long-term use over several decades and that the term "product" is more acceptable to the public than is "chemical". This terminology helps keep in focus for the community that the larvicide products are considered to be biological products (commonly referred to as "biorational") and therefore are quite specific for the control of mosquitoes. There is a noisy but effective minority who ask questions about chemical use. Mosquito control programs answer their questions with information about mosquito biology and ecology, the control products used and their safety profile.

There is a significant "Chemtrail-like" situation with respect to chemicals used in mosquito control. Chemtrail has its origin in the supposed conspiracy theory that aircraft condensation trails contain toxic substances (Wikipedia 2021). When applied to mosquito control, it is a critical issue that emerges every few years. Mosquito control programs must respond to all inquiries, even those that are not core business to discuss the use of helicopters, products or areas where the programs operate. Of course this takes time thus taking staff away from conducting their essential core business duties. However, it is impossible to override the beliefs of some individuals especially those who are utterly convinced on a particular topic which can thus lead to a frustrating endless circle of responses.

Public expectation is a recurring theme in that the local governmental councils have the responsibility (and the public has the expectation) that mosquito control efforts will be made to reduce/minimize product/chemical use by using the materials efficiently. There is a public understanding that mosquito control programs will do everything possible to achieve the goal of keeping mosquito numbers as low as possible. People tend to build up expectations about adulticiding and so it is always carried out in a manner to minimize public concerns. "When spraying, we need community acceptance because we *have* to make a treatment." On the other hand, "public perception is critical – we could be caught short if there were to be a mistake" and "there are ramifications if we get it wrong".

There are some people who are sensitive to chemicals and reluctant to accept them. The opinions of such individuals have been a strong consideration over the last 5 years across all council operations. Notifications are provided prior to aerial treatments from a safety perspective (*e.g.*, helicopters operating close to construction sites or projects). Mosquito management programs are highly visible both via the actions they take and also when there is an increase in mosquito activity. It is critical to the success of the program that communication is effectively provided to our communities so they can understand the balance that programs are working to achieve.

In summary, public perceptions can derail a program if based on misinterpretations so they must be addressed as soon as possible. If these misconceptions are allowed to persist and be spread on social media, it is a "looming threat" by which "we all may be doomed". We have to be able to provide accurate information early, through a multitude of communication channels, so as to be able to address an issue before it takes hold. This is the case for mosquito control as it is for other governmental topics.

CHALLENGES WITH CURRENT TOOLBOX

As most readers are aware, the mosquito control industry is rather small in comparison to other entomological control efforts (*e.g.*, agriculture, urban) making it economically difficult for new products to make their way into the mosquito control "toolbox". However, given the challenges that are faced moving forward, having at least several different larvicides and adulticides at our disposal is important to ensure that appropriate treatments can be made. Over the years we have seen that to accomplish this takes cooperation between mosquito control programs, industry and the research community. It is likely that this sort of arrangement needs to continue into the future. This is especially apparent as mosquito control agencies will need to respond to the problems posed by new mosquito species and pathogens within their jurisdictions.

SOME U.S. PERSPECTIVES. The U.S. mosquito control market is, to a degree, different from most markets in the chemical business. Although our roles differ greatly, the mosquito control community is quite inclusive and considers industry to be a stakeholder. Industry thus is able to play a supporting role in protecting public health and to design products based on stewardship and sustainability.

We all understand that mosquito control programs are unique and have evolved in response to historical events, environmental sensitivity, local mosquito species, and public perception. When visiting a mosquito control program, industry representatives typically request that they explain their program so that the representative can better understand how the program operates. The "why" usually proves to be the most interesting part. This is where a better understanding of local needs, problems, and issues can be gained and then they can be prioritized nationally. The goal of industry is to create products that are efficacious and environmentally sustainable nationally/globally but must also be diverse and flexible enough to become a local solution for each end-user. If industry is doing its job in designing mosquito control products, decisions should be driven by good science as opposed to good marketing.

Every product concept that is proposed goes through some type of ideation process; proof of concept, market analysis, efficacy testing, determining formulations, determining proper use. An immense amount of time and work goes into a product idea before a proposed label is ever submitted to the U.S. Environmental Protection Agency (EPA). The EPA then makes suggestions or requires additional testing to address issues or concerns.

A label is typically submitted and modified numerous times before it receives an EPA registration number.

What does it take to bring a new product to market? If a novel molecule is being considered that has no efficacy data or regulatory history, an investment of $200-$400 million dollars over nearly a decade is likely necessary to assemble the data and finalize a registration. A new molecule must have potential for global impact use across multiple markets to be considered for this level of investment. Molecules or active ingredients that are currently used for other applications can have a smoother and quicker path to market alone. However, there is still significant expense involved in getting through this process. The public health market, of which mosquito control is a part, does not have the volume to support this level of investment. In some favorable situations, a new or existing chemistry can be used for public health and can then justify the additional expense required to add mosquito control applications to the label. Even then, it may take 1-3 years to assemble the required data to submit for registration. Once it becomes a legally registered product for mosquito control, additional years are required for revenues to achieve a sustainable level. Only then does a company begin to recoup its investment and show a profit. It is easy to view companies as greedy and profit driven. However, producing new products must be sustainable for there to be a future of innovation.

In recent years, the mosquito and vector market has benefitted from improved technology and equipment. Short term, our greatest opportunities for innovation are in the application of existing products. One of the things that is important within the mosquito control industry is that it is an open-source community. Districts that have made innovative changes in equipment and strategy willingly share them with others. Industry members are pleased to collaborate with mosquito control programs to expand our knowledge base. Collectively we need to share the challenge to apply products in a better manner and achieve success in a more sustainable fashion.

Obviously, sterile insect techniques (SIT)/genetically modified organisms (GMO)/traps and those companies and scientists that are performing the necessary research to make these viable tools, are extremely important to the future of mosquito control. What is unfortunate is the lack of follow-through funding from some elected officials who are aware that the mosquito control industry is small and the toolbox is shrinking. Federal legislation has passed, and funding has been authorized, to address these existing financial shortfalls but none of the funding has been formally allocated (technically known as "appropriated"). Providing resources to develop these tools for the aforementioned initiatives would go a long way in addressing this problem. The U.S. has already developed a list of the "Pests of Public Health Significance" and appropriating funds for the control of these pests is essential to maintain the health and quality of life we enjoy in the U.S.

SOME AUSTRALIAN PERSPECTIVES. Historically mosquito control products are typically first developed by industry for agricultural or urban pest applications. Control technology is effective with liquid/sand products currently working well. Companies providing aerial application services are world class with their sophisticated equipment, GPS/GIS capabilities and providing post-treatment data as it applies to coverage.

Combination products (such as a *Bti*/methoprene mix) are available but are expensive and suitable only for small areas. *Bti* is working well for larviciding needs, so currently there is not an urgent need for other options that likely will be more expensive. A significant concern is that of product stability while being stored (heat is problematic but suppliers currently pay close attention to this concern), receiving the products that we need in a timely fashion and having a diversity of them available. Mosquito control programs are in the position of having to trust in the suppliers' delivery timetables and with COVID-19, we have seen how outside forces can interfere with supply chain stability. Looking toward the future, interest continues with how new product development is progressing and Australia's Mosquito and Arbovirus Research Committee is paramount to properly accomplishing this need (Dale et al. 2008).

REGULATIONS

Changing regulations relative to pesticide use can greatly hinder a program's effectiveness. New designations to the Endangered Species List in the U.S. or the Environmental Protection and Biodiversity Conservation Act in Australia may make it very difficult, if not nearly impossible, to make timely mosquito control treatments thus in some situations compromising human health. A balance needs to be achieved between the need for mosquito control operations use of pesticides and environmental concerns that deserve due consideration.

SOME U.S. PERSPECTIVES. While the Endangered Species Act (ESA) has been law since 1973, it wasn't until recent years that more insects (especially pollinators) have been listed as threatened or endangered species. Since chemical formulations are designed to control insects of their specific target size, this can be problematic for mosquito control, in particular in regard to adulticiding. While in most cases the timing of control operations and the relatively low dosage of the applications provide a significant margin of error thus minimally impacting

non-target organisms, definitions within the ESA create problems that are becoming more unavoidable. For example, the term “take” (Section 3(18) of the ESA) means to “harass, harm, pursue, hunt, shoot, wound, kill, trap, capture, or collect, or to attempt to engage in any such conduct." The term “harm” is an act which actually kills or injures wildlife. Such acts may include significant habitat modification or degradation when it actually kills or injures wildlife by significantly impairing essential behavioral patterns including breeding, feeding, or sheltering. Both these terms are being broadly interpreted when evaluating mosquito control operations. Treating a field with a mosquito control product that is used by a pollinator could be identified as “harming” or “impairing essential behaviors” if that pollinator happens to be listed, even if the treatment is conducted when none are present.

The ESA, unless amended to address public health mosquito control efforts, will require agencies to be more targeted in control measures. In many circumstances, barrier treatments (except those conducted on buildings or artificial surfaces) will likely have to be reevaluated thus resulting in more frequent and expensive applications in hard-to-reach areas.

As an aside, a discussion topic that has arisen many times is: If mosquito control agencies are able to reduce the population of a mosquito species to near extinction, would mosquito control programs then be prohibited from undertaking ongoing efforts to control that species? Based on the current definition of the law, it appears we would not be able to do so, meaning that we as humans (at least in the U.S) are legislating a situation where potentially millions of people world-wide could die annually from the bite of a particular mosquito species that we otherwise might be able to someday eradicate.

SOME AUSTRALIAN PERSPECTIVES. We are comfortable with current regulations pertaining to mosquito control. The legislative response to mandatory mosquito control is to make certain that a program exists; the products solve 80-90% of the problem and are applied by licensed operators within council under the Pest Management Act Licensing Act 2001. The products are well accepted both by the scientific and general community. From an environmental perspective, we are mindful of the program footprint; for instance, shorebird distributions are taken into account in the Moreton Bay Marine Park area and permits are in place for all standard operations.

MOSQUITO RESISTANCE

Back in the 1950s and 60s, through the use of DDT both as a mosquito larvicide and adulticide, the world saw mosquitoes rapidly become resistant to this and other similar chlorinated hydrocarbon chemicals. Over the ensuing 60+ years, many mosquito species have become resistant to other pesticides as well. This is making for a challenging situation for some mosquito control programs both from a mosquito nuisance and disease-transmission standpoint.

SOME U.S. PERSPECTIVES. Mosquito resistance is clearly a problem that will likely worsen over time. While the resistance to DDT was greatly hastened by its overuse through both larviciding and adulticiding, the same practices are occurring in some locations today. The use of barrier treatments by private pest control operators, as well as some mosquito control agencies, is likely to result in increased resistance of some of the products that are currently available. Where possible, becoming more targeted in our applications will help to slow the spread of this problem.

An easier method to determine pesticide resistance in mosquito populations is needed. For instance, a dipstick-type method (testing pooled mosquitoes) might be a goal which could potentially be employed at most programs. Also a means to better translate lab findings to field situations is needed. Currently this is an intensive endeavor to accomplish which is beyond the capabilities of many mosquito control programs. Naturally when that information becomes available, the problem remains what to do when resistance has been detected. Along with the paucity of novel control strategies, this a powder keg-like situation with the long fuse already lit.

SOME AUSTRALIAN PERSPECTIVES. Fortunately, so far mosquito resistance to products being used has not been a problem in Australia. This is related to the fact that treatment does not completely eliminate mosquitoes so there is little pressure to develop resistance. However if resistance to a product is suspected, we will need the assistance of industry to try and determine if it is real and how to address the problem. Meanwhile, everyone relies on the suppliers “to drop a good product on our doorstep” so that control actions remain effective.

EXOTIC VECTORS AND PATHOGENS

Over the past 20+ years in Florida, several new pathogens and mosquito species have migrated into our areas of jurisdiction. While the mosquito control profession is largely made up of environmentalists at heart, a mandate to control mosquitoes is at the core of our professional mission which at times can make for difficult decisions in dealing with environmental concerns, especially during periods of public health concern.

SOME U.S. PERSPECTIVES. This is a situation which is not new to Florida or California. Indeed, much of

the U.S. is seeing the migration of "new" mosquito species to areas where they had never occurred previously. *Aedes aegypti, Ae. albopictus*, and *Ae. notoscriptus* are all new to S. Cal within the last 20 years, and *Ae. aegypti* has apparently become established in N. Cal. Also, other regions of the country are seeing these species travel much further north than what was expected.

Some agencies in S. Cal, are focusing on public outreach and teaching people what they can do to help curb the spread of new invasive mosquitoes. While agencies are exploring adult mosquito control interventions such as genetically modified organisms (GMO) or sterile insect techniques (SIT), adulticiding does not seem to be a tool readily used or available. Based on the past few decades, there is every reason to believe that this influx of exotic mosquitoes to the U.S. from other countries will continue and perhaps hasten as climate change continues to impact our planet and that the use of chemicals to try and limit their penetration into new areas will be important.

SOME AUSTRALIAN PERSPECTIVES. Currently Australian mosquito control programs are not set up to respond to the incursion of exotic mosquitoes or pathogens. If that should occur, it would likely be categorized as a "disaster response". Additionally, training and surveillance of personnel would be needed and that would need to be resourced. The Australian Quarantine and Inspection Service monitors points of entry for foreign exotics and is assisted by local mosquito control if any are detected (*e.g., Aedes albopictus).* Within Queensland, the distribution of *Ae. aegypti* is monitored in case this species moves south towards Brisbane.

LOOKING FORWARD

We recognize that much of the information presented in this paper has been previously discussed informally and at local, state, national, international meetings. However, we hope that being presented in this fashion will stimulate further conversations and lead to actions which will further stimulate the mosquito control profession to be able to accomplish their mandates for the future. Mosquito control rightfully has the reputation of being frugal and imaginative and doing well with what is provided via the political process. However, as time passes and some of the issues mentioned above become more problematic, having adequate resources will be important in keeping our profession at the forefront of public health in the U.S., Australia and worldwide.

Mosquito control professionals must be mindful of ways that climate change will likely alter the ways our work needs to be carried out. Some of these weather-related events can have huge impacts on mosquito populations whether they are nuisance mosquito outbreaks or ones of mosquito-transmitted disease importance. Mosquito control agencies must be flexible in responding to such challenges and the use of larvicides and adulticides will undoubtedly continue to be a large part of the control efforts. Mosquito control needs to be especially adept at interpreting meteorological information. Recently in Queensland, in regard to weather forecasting, a member of the public said to mosquito control officials "I trust you above everybody else" which was taken as a great vote of confidence in their forecasting abilities (based on meteorological data that they receive). Such compliments make the efforts of mosquito control professionals highly rewarding. Dealing with problem solving and the logistical aspects of control actions can be an enjoyable part of the work along with daily challenges of never knowing what will come across an employee's desk.

Interactions amongst mosquito control workers, industry, and academic scientists are vital to the profession moving forward in a positive fashion given the many challenges that are being faced. In Australia, such a coordinated effort is demonstrated through efforts of the Contiguous Local Authorities Groups (CLAGS) and in Florida through the Florida Coordinating Council on Mosquito Control (Dale et al. 2008). Other opportunities to share information and work together include the American Mosquito Control Association, the Mosquito Control Association of Australia, and many regional and state association meetings. With this said, we remain confident that the use of chemicals for mosquito control will continue to be an important component of most IMM programs as we move through the 21st Century. Decisions made when using them will need to continue to consider potential environmental impacts as part of their use profile.

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RESISTANCE AND RESISTANCE MANAGEMENT OF BIORATIONAL LARVICIDES FOR MOSQUITO CONTROL

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ABSTRACT

Mosquitoes and mosquito-borne diseases remain a significant threat to public health and the well-being of humans and animals. Often mosquito control is the only feasible way to combat mosquito-borne diseases. Biorational mosquito larvicides based on microbials and insect growth regulators (IGR) have been playing an irreplaceable role in integrated mosquito control worldwide. While the relative target specificity, non-target safety and environmentally friendly profile are well recognized in biorational larvicides, their risk of resistance and cross resistance must be taken into consideration in mosquito control operations. This paper provides a review of the resistance risk, historical and current status, and management tactics for the commonly used mosquito larvicides such as *Bacillus thuringiensis* subsp. *israelensis (Bti)*, *Bacillus sphaericus*, spinosad, methoprene, pyriproxyfen, and diflubenzuron. *Bti* poses the lowest risk of resistance and plays a unique role in resistance management. Various levels of resistance to *B. sphaericus* have been reported in both laboratory and field populations during the past decades worldwide. High level of resistance to spinosad has been documented recently in laboratory populations of *Culex quinquefasciatus*, followed by preliminary report from field populations of *Cx. pipiens*. As to resistance to IGRs, documentations on laboratory and/or field populations have become available since the early 1970s for methoprene and the 1990s for pyriproxyfen. The most recent report on resistance to diflubenzuron reconfirmed the earlier studies. The tactics to prevent resistance and restore the susceptibility in mosquitoes to these biorational larvicides have been developed and implemented in some cases, which is crucial to sustainable integrated mosquito management.

Key Words: Microbial larvicides; Insect growth regulators; Mosquito control; Resistance; Resistance management

INTRODUCTION

Mosquitoes and mosquito-borne diseases pose a significant public health threat and economic burdens worldwide, particularly to the countries in tropical and subtropical regions. Upon globalization, demographic growth, and subsequently environmental impact, public health concerns created by mosquitoes have been on the rise despite diligent efforts of integrated mosquito control programs. Often, mosquito control is the only effective and feasible way to combat mosquito-borne diseases, where larviciding to target aquatic immature stages is often the primary intervention. However, the availability of effective, environmentally friendly, and non-target safe and affordable larvicides is very limited today. Thís situation has been worsened by strict regulations, high cost in development and registration, narrow market niche of products, emergence, or resurgence of new vector species and associated diseases and lastly, development of resistance. To achieve sustainability in mosquito control, resistance management with the limited available control tools must be integrated by mosquito control operations. The current paper is considerably concentrated and updated from the previously published book chapters to facilitate the need of field mosquito control professionals The audience who are interested in details of this topic can refer to Su (2016a,b).

BACILLUS THURINGIENSIS SUBSP. *ISRAELENSIS (BTI)*

The entomopathogenic *Bacillus* was identified in 1901 from silkworm that suffered the sotto disease and was named *Bacillus sotto*. However, the finding of this *bacillus* in 1911 from Mediterranean flour moth *Anagasta kuehniella* caterpillars lead to the official name of *Bacillus thuringiensis* (Roh et al. 2007). To date, at least 70 serotypes, with more than 80 subspecies have been identified, among which 14 serotypes and 16 subspecies show lethal activities against mosquito larvae. *Bacillus thuringiensis* subsp. *israelensis* (*Bti*), serotype H-14, was discovered in Israel in 1976 (Goldberg and Margalit 1977, Margalit and Dean 1985). Four endotoxins including cytolytic toxin Cyt 1A and crystal toxins of Cry4A, Cry4B, Cry 11A are produced during sporulation (Tabashnik 1992, Wirth et al. 2004), which are activated by enzymatic proteolysis at a high pH environment in the mosquito midgut. *Bti* is categorized as a Group 11 pesticide, i.e., microbial disruptor of insect midgut membranes by Insect Resistance Action Committee (IRAC) (Su 2016a). *Bti* is registered as biopesticide by the

US Environmental Protection Agency (US EPA) in 1982 (Wang et al. 2018a).

Numerous studies have been attempted and published about induction of larval resistance to *Bti* in *Culex pipiens* complex or *Aedes aegypti* since 1983. Response to sublethal exposure for numerous generations, tolerance or very low, unstable resistance was developed (Vasquez-Gomez 1983, Goldman et al. 1986, Saleh et al. 2003, Mittal 2005, Su 2016a). However, the cryptic *Bti* resistance in field *Aedes* populations was detected to crystal toxins in response to previous exposures to whole *Bti* when tolerance or low-level resistance has developed (Tetreau et al. 2012, 2013). In field populations, the risk of resistance development to wild type *Bti*, i.e., the intact toxin complex, is very low. The extensive use of *Bti* products to control floodwater mosquitoes *Ae. vexans* over an area of approximately 500 km^2 for more than 36 years in the Rhine River area in Germany has been systematically documented, no noticeable reduction in susceptibility was detected (Becker et al. 2018). Low levels of resistance were noticed in *Cx. pipiens* complex populations in different geographical locations where *Bti* products were used for different periods of time (Wirth et al. 2001, Vasquez et al. 2009), but these levels of resistance did not cause much concern. One field study however, reported that collections from Syracuse and Albany, New York showed 33-41- and 6-14-fold resistance, respectively, the test material was laboratory cultured strain ISP-80 (Paul et al. 2005). It is worthwhile to follow the resistance status in these populations. Exposures to individual crystal toxins of *Bti* are conducive to resistance and cross resistance development among the toxins, in the absence of Cyt1A toxin, highlighting the importance of the full combination of toxins found in wild *Bti* in resistance management (Georghiou et al. 1997, Wirth et al. 1997). Cyt1A from *Bti* does not possess significant larvicidal activity alone, but plays a critical role in overcoming, preventing, and delaying resistance development to Cry toxins, partially since Cyt1A functions as a receptor to enhance the binding of the crystal toxins (Chueng et al. 1987, Pérez et al. 2007).

BACILLUS SPHAERICUS

To date, over 300 strains of *B. sphaericus* belonging to 49 serotypes have been identified, among which 16 strains, 9 serotypes showed various levels of activity against mosquito larvae. The following strains possess high mosquitocidal activity - 2362, 1597, 2297, C3-41 and IAB-59, among which the strain 2362 was isolated from adult blackfly *Simulium damnosum* in Nigeria in 1984 and was extensively studied and developed. Active strains produce parasporal inclusions during sporulation, which contains crystal binary toxins. Some strains also synthesize non-crystal mosquitocidal toxins (Mtx) during the vegetative growth phase. The mode of action of the binary toxins is somewhat similar to *Bti* toxins. The receptor of the binary toxins is a 60 kDa α-glucosidase, which is anchored in the mosquito midgut membrane via a glycosyl-phosphatidylinositol (GPI) anchor. While belonging to the same IRAC group as *Bti*, *B. sphaericus* has a narrower species spectrum. Some *Aedes* spp., for example *Ae. aegypti* and *Ae. Albopictus*, are much less susceptible than *Culex* spp. to this microbial agent (Su 2016b). *Bacillus sphaericus* strain 2362 was registered as biopesticide by the US EPA in 2000 (Wang et al. 2018a).

Various levels of resistance to *B. sphaericus*, mostly strain 2362, in laboratory colonies of *Cx. pipiens* complex, has been reported in different countries since 1994 as a result to sublethal exposure for different periods of time (Rodcharoen and Mulla 1994, Wirth et al. 2000, Pei et al. 2002, Amorim et al. 2007, Zahiri et al. 2002, Zahiri and Mulla 2003). It appeared that the resistance evolution to *B. sphaericus* in response to laboratory selection depends on genetic background, selection procedures, and other unknown factors. Resistance level is also dependent on the susceptibility of the reference population tested. The resistance to *B. sphaericus* is stable in absence of selection pressure (Amorim et al. 2010). As to the cross resistance among different strains, once mosquitoes develop resistance to a given strain of *B. sphaericus*, they are also often resistant to other strains because of the similarity of the binary toxins in most strains. Fortunately, mosquitoes that have developed resistance to various strains of *B. sphaericus* remain susceptible to *Bti* (Wirth 2010, Su 2016a). The cross resistance among different strains is mild between the strains that also produce the Mtx (Yuan et al. 2003). The Mtx from some *B. sphaericus* strains not only enhance the larvicidal activity of *Bti* Cry toxins, but also mitigate resistance development to Cry toxins (Wirth et al. 2014). These results indicated the potential role of Mtx in resistance management to *Bti* and *B. sphaericus*.

The earliest resistance to *B. sphaericus* in field populations was reported in *Cx. pipiens* in southern France where the resistance ratio at LC_{50} was 70-fold because of extensive field applications (Sinègre et al. 1994). Numerous reports on resistance have been published since then in the *Cx. pipiens* complex from different countries (Su 2016a). The highest level of resistance was documented in a *Cx. quiquefasciatus* population in Thailand, where *B. sphaericus* was used for only 4 months with 5 treatments (Mulla et al. 2003). The resistance levels at LC_{50}, depending on reference colonies, were 21,100-28,100-fold against commercial product or > 125,000-200,000-fold against technical-grade material (Su and Mulla 2004). Two cases

on high levels of resistance to *B. sphaericus* in the USA, where *B. sphaericus* products-based strain 2362 have been applied for various time, were reported in wild populations of *Cx. pipiens* in California and Utah (Su et al. 2018, 2019). In the *B. sphaericus*-resistant population from California, various levels of resistance or tolerance were also noticed to abamectin, pyriproxyfen, permethrin and indoxacarb. However, it would not be feasible to determine they are cross- or independent multiple resistance due to unknown field exposures (Su et al. 2018). The resistance evolution in response to field application of *B. sphaericus* products varies greatly, depending on exposure to naturally existing strains, population genetic background, gene exchange with untreated populations, as well as product application strategies. As to the mechanism of resistance to *B. sphaericus*, it is mostly believed that recessive genes are involved. Although various theories have been proposed, lack of specific binding of binary toxins to α-glucosidase receptors in the midgut appeared the main reason, which is due to the partial deletions of the gene that encodes the receptor (Su 2016a).

Beside conventional practice for resistance management, *Bti* can be used as a powerful tool to mitigate resistance to *B. sphaericus*. Before it occurs, resistance to *B. sphaericus* can be delayed or prevented by the mixture of *Bti* and *B. sphaericus* because of the synergistic action among total 6 toxins (Cyt 1A, Cry4A, Cry4B, Cry 11A from *Bti* and binary toxins from *B. sphaericus*), particularly the presence of Cyt1A (Wirth 2010). While rotation of two pesticides with different modes of action can be commonly used for resistance prevention, the rotation of *B. sphaericus* and *Bti* surprisingly resulted in much higher levels and faster emergence of resistance as compared with *B. sphaericus* alone for the unknown reasons. However, selection with mixtures of *Bti* and *B. sphaericus* almost negated emergence of resistance to *B. sphaericus* (Zahiri and Mulla 2003). Recently, the recombinant that produces toxins from both *Bti* and *B. sphaericus* provides another path for not only mitigation of resistance also enhancement of lavicidal activity and efficacy. Combination of *B. sphaericus* with botanical pesticides such as azadirachtin also provided a potential to mitigate resistance development to *B. sphaericus* (Poopathi et al. 2002). The susceptibility to *B. sphaericus* in a resistant colony was partially restored by *Bti*, and rotation or mixture of *Bti* and *B. sphaericus* (Zahiri et al. 2002). In field operations, highly *B. sphaericus*-resistant mosquitoes can be effectively controlled by *Bti* alone or through a combination of *Bti* and *B. sphaericus*. At the same time, the lost susceptibility to *B. sphaericus* can be restored upon time by new interventions applied (Yuan et al. 2000, Mulla et al. 2003, Su et al. 2018, 2019b). The *B. sphaericus* resistant mosquitoes might carry some fitness disadvantages, but there seemed not to be any difficulties in sustaining the population integrity (Rodcharoen and Mulla 1997, Amorim et al. 2010).

SPINOSYNS

Spinosad, consisting of spinosyn A ($C_{41}H_{65}NO_{10}$) and D ($C_{42}H_{67}NO_{10}$) in the ratio of 85% and 15% respectively, is produced by a naturally occurring, soil-dwelling actinomycete, *Saccharopolyspora spinosa*, which acts as a nicotinic acetylcholine receptor (nAChR) allosteric modulator. Spinosad, along with spinetoram that consists of spinosyn J and L, is categorized as a Group 5 insecticide by IRAC, and registered as an organophosphate alternative/reduced risk pesticide by the US EPA in 1997 (Wang et al. 2018b).

Spinosyns exert pesticidal activity after ingestion and cuticle absorption against a broad spectrum of susceptible insect species by stimulating nACh and γ-aminobutyric acid (GABA) receptors and causing rapid excitation of the insect nervous system. As a relatively new product for mosquito control, studies to evaluate resistance development risk and resistance management strategies for spinosyns are rather rare. The first attempt was made for *Cx. quinquefasciatus* where a selection pressure was applied at $LC_{70\text{-}90}$ levels to late 3rd and early 4th instar larvae in each generation in a laboratory colony. Resistance increased gradually to 1,415.3- to 2,229.9-fold at LC_{50} and 9,613.1- to 17,062.6-fold at LC_{90} at after selection for 45 generations. The exponential elevation of resistance levels throughout selection indicated that a recessive mechanism might have been involved during resistance development to spinosad (Su and Cheng 2012, 2014a). This "recessive mechanism" was indicated later by a two-way cross test between males and females of the resistant and susceptible populations, where high levels of resistance disappeared at F_1 (Su et al. unpublished). Regardless of the high-level resistance, the bio-fitness cost seemed very minimum as the colony has propagated well under standard maintenance protocols. The resistance to spinosad tended to decline in the absence of selection pressure and more so if with simultaneous infusion of susceptible individuals. The resistance declined faster when existing resistance was at the lower levels than at the higher levels (Su et al. unpublished).

There was a lack of cross resistance to the following pesticides in this highly spinosad-resistant *Cx. quinquefasciatus*: *B.t.i.*, a combination of *B.t.i.* and *B. sphaericus*, methoprene, pyriproxyfen, diflubenzuron, novaluron, temephos or imidacloprid. However, it did show various levels of cross resistance to *B. sphaericus*, spinetoram, abamectin and fipronil. On the other hand, a long-term laboratory colony of *Cx. quinquefasciatus* that

is highly resistant to *B. sphaericus* (Wirth et al. 2000), was as susceptible as a laboratory reference colony to spinosad and spinetoram, indicating a one-way cross resistance from spinosad to *B. sphaericus*. Field-collected and laboratory-selected *Cx. quinquefasciatus* that were resistant to methoprene, did not show cross resistance to spinosad and spinetoram (Su and Cheng 2014b). Currently, there is a lack of research on resistance management strategies pertinent to spinosad. Preliminary studies indicated that *Bti* plays a unique role in spinosad resistance management. Treatment by *Bti* for 15 generations almost completely restored the susceptibility to spinosad in a highly spinosad-resistant laboratory population (Su et al. unpublished).

As to the field monitoring on resistance in mosquitoes to spinosad, data is quite meager. Recent report has indicated the occurrence of spinoad resistance in field populations of *Cx. pipiens* in urban northern California (Wheeler et al. 2022). Further monitoring is hence highly recommended.

INSECT GROWTH REGULATORS

Methoprene, a true juvenile hormone analog, interrupts juvenile hormone balance during the transition from late 4th instar larvae to pupae and adults. Most mortality occurs at pupal stage or incompletely emerged adults. This synthetic compound was categorized as Group 7A by IRAC and registered as biopesticide by the US EPA in 1975 (Wang et al. 2018a). The earliest laboratory studies on resistance development in mosquitoes dates back to early 1970s, when the collective results indicated low risk of resistance development (Su 2016a). One recent study showed that the resistance level was significantly elevated by continuously exposing field collected *Cx. quinquefasciatus* that had low level of existing resistance to methoprene for 30 generations. At this time, various levels of cross resistance to other commonly used pesticides were revealed in the selected population. Cross resistance to *B. sphaericus* was the most profound, amounting to 77.50- to 220.50-fold. This cross resistance seemed only one-way from methoprene to *B. sphaericus*, as *B. sphaericus*-resistant mosquitoes remained susceptible to methoprene (Su et al 2018, 2019b).

As to resistance development in wild populations of mosquitoes, data are quite limited mostly due to lack of monitoring. The first report in this regard was published in 1998, when an *Ae. taeniorhynchus* population in Florida showed 15-fold resistance after applications of a methoprence product during 1989 to 1994 (Dame et al. 1998). Methoprene tolerance in *Ae. nigromaculis* was discovered in central California after 20 years of treatment by methoprene products, followed by a control failure during 1998-1999 (Cornel et al. 2000, 2002). The documented resistance seemed not related to the metabolic detoxification by P450 monooxygenase and carboxylesterase, and treatments using *Bti* partially and gradually restored the susceptibility to methoprene (Cornel et al. 2002). Other reports on field populations showed varying and moderate levels of resistance, such as 4.7-16-fold in *Cx. pipiens* in Cypress (Vasquez et al. 2009), 9-54-fold in *Cx. quinquefasciatus* in southern California (Su and Cheng 2014, Su et al. 2021), and elevated resistance levels in *Cx. pipiens* in northern California (Wheeler et al. 2022).

The juvenile hormone analog mimic pyriproxyfen was synthesized in the early 1970s, the IRG activity of which is much higher than methoprene (Su and Cheng 2014, Su et al. 2018, 2019a, b). Pyriproxyfen has the identical activity to juvenile hormone III (JH III) in mosquitoes as does methroprene, but is not structurally related to JH III, which is the opposite of methoprene. This compound was categorized as Group 7C by IRAC and registered as organophosphate alternative/reduced risk pesticide by the US EPA in 1998 (Wang et al. 2018b). Limited data showed very low risk of resistance in mosquitoes (Schaefer et al. 1991) until one report was published (Su et al. 2019a) that showed a noticeable level of resistance in a field population of *Ae. aegypti* in southern California. It is unlikely that this field-occurred resistance is caused by public health applications, as there was no record of such application up to collections of samples for testing. This pyriproxyfen-resistant *Ae. aegypti* did concurrently show low level resistance to methoprene which possesses the similar mode of action (Su et al. 2019a). Assuming that this low-level methoprene resistance is caused by exposure to pyriproxyfen, there might be a two-way low level cross resistance between methoprene and pyriproxyfen, when connecting this finding with the cross-resistance profile in methoprene-resistant *Cx. quinquefasciatus* populations (Su et al. 2021).

Chitin synthesis inhibitors such as diflubenzuron have a very limited use in the USA. This compound is a non-selective chitin synthesis inhibitor which interrupts formation of the exoskeleton, interferes with integrity of cuticle, and leads leakage of body fluid and ultimately mortality of a wide variety of target organisms. It acts on the entire life cycle, particularly younger larvae which show higher susceptibility than other stages. This compound was categorized to Group 15 (Inhibitors of chitin biosynthesis affecting CHS1) by IRAC and registered as organophosphate alternative/reduced risk pesticide by the US EPA in 1998 (Wang et al. 2018b). To date, most studies on resistance management are limited to laboratory populations and results point to

low risk of resistance (Su 2016a). However, high levels of resistance to diflubenzuron were identified very recently in *Cx. pipiens* populations from Italy (Grigoraki et al. 2017, Porretta et al. 2019) and Turkey (Guz et al. 2020). This resistance was associated with mutations at amino acid I1043 (I1043F, I1043M, and I1043L) of the chitin synthase gene. The contribution of these mutations to diflubenzuron resistance was validated by introducing them to the *Drosophila melanogaster* chitin synthase gene, where I→M mutation results in a >2,900-fold and the I→L mutation a >20-fold resistance (Grigoraki et al. 2017, Porretta et al. 2019, Fotakis et al. 2020, Mastrantonio et al. 2021).

CONCLUSIONS

In summary, while the need for mosquito larvicides is on the rise due to the emergence and resurgence of vectors and vector-borne diseases, their availability unfortunately is at the lowest point for numerous reasons. Resistance to the limitedly available larvicides creates further challenges for mosquito control operations. Among the advantages of *Bti*, minimum risk of resistance evolution due to the intact endotoxin complex, synergism among individual toxins and presence of Cyt1A, make this microbial agent a unique tool in controlling mosquitoes, blackflies, and midges. More importantly, *Bti* seems to be a critical tool in resistance mitigation to other biorational larvicides, including delaying resistance evolution before the fact and restoring susceptibility after the fact. While appreciating the values of *B. sphaericus*, its toxin simplicity, along with previous exposure to wild strains in nature and the genetic background of larval populations, collectively lead to a noticeable level of risk in resistance development. Combining *Bti* and *B. sphaericus* deems many benefits in resistance management and efficacy enhancement. Based on limited data, it is not recommended to rotate *Bti* and *B. spahaericus* to delay resistance development to *B. sphaericus*, although more studies are needed to elucidate the unknown mechanism. Larval mosquitoes develop resistance to spinosad quickly if resistance management tactics are not implemented strategically, largely due to the mode of action of these neurotoxins and chances of sub-lethal exposures, which has been well documented in agricultural pests. Tactics to prevent, or at least delay resistance development, and to restore spinosad susceptibility after resistance development in mosquitoes, should be developed and implemented. The overall risk of resistance development to methoprene is low when one reviews the historical cases over decades of applications. However, due to the narrow window of susceptibility, i.e., the transition period from late 4th instar larvae to pupae and adult emergence, sublethal exposure, the leading cause of resistance development, is unavoidable when treating larval populations with mixed stages, as young larvae have a high lethal level as compared to older ones. It is generally believed that pyriproxyfen has low resistance risk because of its strong growth regulation and other activities against various life stages. However, its persistence in the environment could lead to sublethal exposure, hence development of tolerance and resistance. It is a surprise to see the recent documentation of resistance to diflubenzuron in *Cx. pipiens*. As a chitin synthesis inhibitor with a broad activity window as compared with juvenile hormone analog or mimic, diflubenzuron is obviously still not resistance proof. Another important point is that mosquitoes have specific exposures of *Bti* and *B. sphaericus* from public health applications only, while the exposures to other larvicides such as spinosad, methoprene, pyriproxyfen, and diflubenzuron, can be undocumented and quite broad from urban, horticulture and agriculture applications. Although often there is a bio-fitness cost in resistant mosquitoes which may bring negative impacts on life events and vectorial capacity of mosquitoes (Su 2016a), the consequences of resistance evolution remain costly.

Considering the widespread occurrence of pyrethroid resistance detected in adult mosquito populations, resistance to biorational larvicides must be monitored and mitigation measures must be implemented to ensure their availability in mosquito management programs.

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AEDES PERTINAX AND *CULEX INTERROGATOR:* TWO MOSQUITO SPECIES NEW TO LEE COUNTY, FLORIDA

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ABSTRACT

Recent introductions of mosquito species new to Florida, and range expansions of other species throughout the state, have many mosquito control personnel on alert for discovery of new species in their area. Adult mosquito specimens collected in traps are a common method of detecting new species, but larval surveillance can be important as well. Larval surveillance in Lee County, Florida has increased over the past two years, and samples collected in the 2021 summer season revealed first-time records of two species new to the county: *Aedes pertinax* and *Culex interrogator*. Morphological and genetic methods were used to identify these two species new to the county. These species may have been present in Lee County in years past, but without these larval samples, they might have continued to go undetected due to their similar appearance to more commonly-occurring species.

Key Words: *Aedes pertinax*, *Culex interrogator*, larval surveillance, Lee County

INTRODUCTION

In recent years there have been many species of mosquitoes detected for the first time in various counties throughout Florida (Blosser and Burkett-Cadena 2016, Burkett-Cadena and Blosser 2017, Riles et al. 2017, Shin et al. 2016). In just the past 20 years, 11 mosquito species were detected for the first time in peninsular Florida (Darsie et al. 2002, Darsie 2003, Darsie and Shroyer 2004, Smith et al. 2006, Shroyer et al. 2015, Shin et al. 2016, Blosser and Burkett-Cadena 2016, Burkett-Cadena and Blosser 2017, Riles et al. 2017, Riles and Connelly 2020, Reeves et al. 2021). One of the more recent arrivals in Lee County is *Culex coronator* (Dyar & Knab), which was first detected in Florida in 2006 (Smith et al. 2006) and later in Lee County in July 2010 (Connelly et al. 2016). This species has since become established in the county, being regularly found in traps. Other recently detected species in Florida such as *Cx. interrogator* (Dyar & Knab) and *Ae. pertinax* (Grabham) have been found in neighboring Collier County but not yet reported from Lee County (Riles and Connelly, 2020). With the recent report by Reeves et al. (2021) on the establishment of *Ae. scapularis* (Rondani) in Miami-Dade and Broward Counties, Lee County Mosquito Control District (LCMCD) was extra vigilant in identifications in 2021 in an effort to detect this and other species new to Lee County.

Normally the LCMCD surveillance department is the first to detect species new to the county when they are captured in traps and identified by biologists. However, with the recent increase in larval collection activities, these species are being detected through the field validation department (FVAL). FVAL is responsible for evaluating the efficacy of new products as well as monitoring the development of resistance against our current arsenal of materials. In order to conduct the various tests, healthy wild adult mosquitoes are tested alongside susceptible colonies. Most often the wild mosquitoes are collected by field inspectors as larvae and then reared to adulthood in the insectary.

Larval collections usually include one of four main species: *Aedes taeniorhynchus* (Wiedemann), *Culex quinquefasciatus* Say, *Culex nigripalpus* Theobald, and *Psorophora columbiae* (Dyar & Knab). Sometimes, however, additional species are mixed in with these samples. In the summer of 2021, two samples had rather distinct looking larvae, which to the naked eye were markedly different from the usual samples. Further investigation revealed that these are species new to Lee County, Florida: *Aedes pertinax* and *Culex interrogator*.

MATERIALS AND METHODS

Larval samples were collected weekly by field inspectors. Live larvae were introduced into the insectary where they were gently rinsed, placed into pans and reared until pupation (80% humidity and 26.7°C, 14:10 light:dark). Larvae were fed daily with a finely ground powder of Mazuri® Rat and Mouse Diet 5663 (St. Louis, MO). Pupae were transferred to clean water and placed into insect cages for emergence. Adults were provided with 20% sucrose solution-saturated cotton pads as a source of carbohydrates.

The first larval sample in question was reared to adulthood for identification. The second sample in question was identified at the fourth instar larval stage, and then reared to the adult stage. Both samples were identified using a stereo microscope under 40X magnification following the keys of Darsie and Ward (2005). A modified couplet was added by LCMCD employees to couplet 51 of the adult *Aedes* key to allow for differentiation between *Aedes atlanticus* (Dyar & Knab), *Aedes tormentor* (Dyar & Knab) and *Ae. pertinax*, following the description of *Ae. pertinax* given in Shroyer et al.(2015). This description recognizes a more narrow and variable scutal stripe of pale scales in *Ae. pertinax*.

Following the morphological identifications, ten adults of each species were placed separately into 1.5 mL microcentrifuge tubes filled with 70% ethyl alcohol and were sent to the Florida Medical Entomology Laboratory (FMEL) in Vero Beach, Florida for molecular confirmation, as these were both species new to the county and morphologically similar to other species known to reside in the county. At FMEL, morphological identifications were confirmed through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene (Hebert et al. 2003). From each specimen, a single leg was removed with flame-sterilized forceps and transferred to a new 1.5 mL tube. DNA was extracted from each leg using the Zymo Quick-DNA Miniprep Plus Kit (Genesee Scientific Corp., El Cajon, CA). Extracted DNA from each specimen was used as template in a polymerase chain reaction (PCR) to amplify a 648 bp fragment of the DNA barcoding region of the specimen's COI gene using the primers and PCR conditions of Hebert et al. (2004). The remaining volume of each PCR product was sent to Eurofins Genomics (Louisville, KY) for one directional Sanger sequencing (Sanger et al. 1977).

Species level identifications were made using the Barcode of Life Datasystems (BOLD; Ratnasingham and Hebert 2007), and by alignment of specimen sequences to sequences derived from *Aedes* and *Culex* reference specimens curated by the Reeves Laboratory molecular collection. Edited sequences were submitted to the BOLD v. 4 Identification Engine for alignment to reference sequences. Sequences from suspected *Ae. pertinax* specimens were aligned and compared to the COI sequences of all North American *Aedes* Protoculex Group (Wilkerson et al. 2015) species, *Ae. atlanticus*, *Ae. dupreei* (Coquillett), *Ae. pertinax*, and *Ae. tormentor*. Sequences from suspected *Cx. interrogator* specimens were similarly aligned and compared to all other *Culex* Subgenus *Culex* species known from Florida. For both groups, neighbor-joining trees were constructed using the Geneious Tree Builder tool in Geneious Prime Version 11.0.6, with the Jukes-Cantor genetic distance model.

RESULTS AND DISCUSSION

Locality and date information for the new detections are shown in Table 1. The first sample of unknown mosquitoes was morphologically identified as *Ae. pertinax*. *Aedes pertinax* is already known to be in Collier County, Florida (Riles and Connelly 2020), immediately south of Lee County, and is morphologically similar to and often confused with *Ae. atlanticus* (Shroyer et al. 2015). These larvae were collected from a coastal flooded woodland which drains into a roadside ditch, both containing freshwater. The sample contained approximately 60 of the unknown larvae and no additional organisms. DNA barcoding confirmed this identification with COI sequences from all specimens in question 98.4-100% similar to reference *Ae. pertinax* sequences, and all sequences from unknown specimens grouping together with *Ae. pertinax* reference specimens in the neighbor-joining tree.

The second sample of unknown larvae was identified morphologically as *Cx. interrogator*, a species which is also known to occur neighboring Collier County and which is morphologically similar to *Cx. nigripalpus* and *Culex restuans* Theobald. This sample was collected from a roadside ditch in an urban area alongside larvae of *Cx. nigripalpus*, *Uranotaenia* spp., and *Anopheles* spp. Non-mosquito arthropods from this sample included copepods, damselfly and dragonfly naiads, and dytiscid beetle larvae. The water also contained string algae. Molecular analysis confirmed the identification of these specimens as *Cx. interrogator*. Sequences derived from all included specimens were 98.2-100% similar to *Cx. interrogator* reference sequences, and together with *Cx. interrogator* reference sequences, formed a clade distinct from all other Florida *Culex* subgenus *Culex* species.

These findings represent the first records of two nonnative species found in Lee County, Florida. As suggested by Shin et al. (2016), it is likely that both *Cx.*

interrogator and *Ae. pertinax* have been in the county for some time but have gone undetected due to their morphological similarities with common native species in the county, namely *Cx. restuans* and *Ae. atlanticus*, respectively. The geographic distribution of *Ae. pertinax* is not well characterized, but the species is native to islands of the Caribbean region, including some Bahamian islands, Cuba, Hispaniola, Jamaica, and Puerto Rico (Belkin et al. 1970, Shroyer et al. 2015). *Aedes pertinax* was first reported in Florida from specimens collected in 2011 in Indian River County, on the Atlantic Coast of central Florida (Shroyer et al. 2015). Adult *Ae. pertinax* are morphologically similar to *Ae. atlanticus* and *Ae. tormentor*, two other members of the *Aedes* Protoculex Group. All three species have a distinct stripe of pale scales along the median of the scutum. In the adults, *Ae. pertinax* differs from *Ae. atlanticus* and *Ae. tormentor* in the width of the scutal stripe, which is substantially more narrow or indistinct than those of the other two species (Fig. 1). See Shroyer et al. (2015) for additional details on distinguishing these species.

Culex interrogator is another recent detection in Florida, first detected by larval sampling in Broward County in 2013 (Shin et al. 2016). Previously, *Cx. interrogator* was known in the United States from south-central Texas and western Arizona (Darsie and Ward 2005) and occurs south through Mexico and Central America (Carpenter and LaCasse 1955), as well as on some Caribbean islands (Menzies et al. 2018, Sosa et al. 2020). *Culex interrogator* has become widespread in Florida, and has been collected in various counties from the Florida Panhandle to the southernmost peninsular counties (Shin et al. 2016, Riles and Connelly 2020). In Florida, *Cx. interrogator* is sympatric with eight other *Culex* subgenus *Culex* species and may be confused with *Cx. restuans* or *Cx. quinquefasciatus* in the adult stage. Adult *Cx. interrogator* (Fig. 2) are generally smaller than other Florida *Culex* (*Culex*) species, have complete basal bands across the abdominal terga, and a pair of dark integumental spots on the thoracic pleura, one each on the meskatepisternum and mesepimeron (a character shared with *Cx. coronator*, *Cx. declarator*, and *Cx. bahamensis*, though this is less distinct in this species). See Shin et al. (2016) for further details on distinguishing the adults and larvae of *Cx. interrogator*.

After detecting these species from our larval surveillance, LCMCD has begun to look more closely at the adults captured in traps and has already found many more *Ae. pertinax* adults spread throughout the county (Fig. 3). We expect that this species has been in the area for quite some time. As we begin to examine the *Culex* adult and larval samples more closely, we expect to see a similar trend. These new findings underscore the importance of larval surveillance and identification as an additional and important avenue for the detection of previously undocumented mosquito species.

Aedes pertinax and *Cx. interrogator* were both detected in Florida only in the past ten years. Since mosquito identification keys are infrequently updated, neither of these two species are included in commonly used resources for the morphological identification of Florida or southeastern United States species. These two mosquito species are morphologically similar to native mosquito fauna with which they may be easily confused. We recommend those involved with mosquito identification familiarize themselves with these and other recently detected Florida mosquito species.

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Table1. Locations, dates and collector identification for the initial larval collections of *Aedes pertinax* and *Culex interrogator* in Lee County.

Species	Location	Date	Site type
Aedes pertinax	26.419839, -81.821178	7/8/2021	Woodland puddle/ ditch
Culex interrogator	26.672734, -81.814886	9/7/2021	Roadside ditch

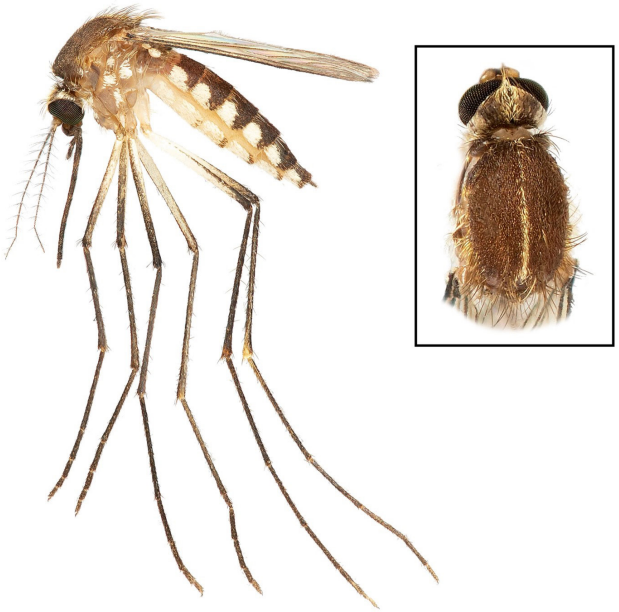

Figure 1. Lateral view of adult female *Aedes pertinax* collected in Indian River County on 2 February 2019. Inset shows narrow scutal stripe of pale scales of the same specimen.

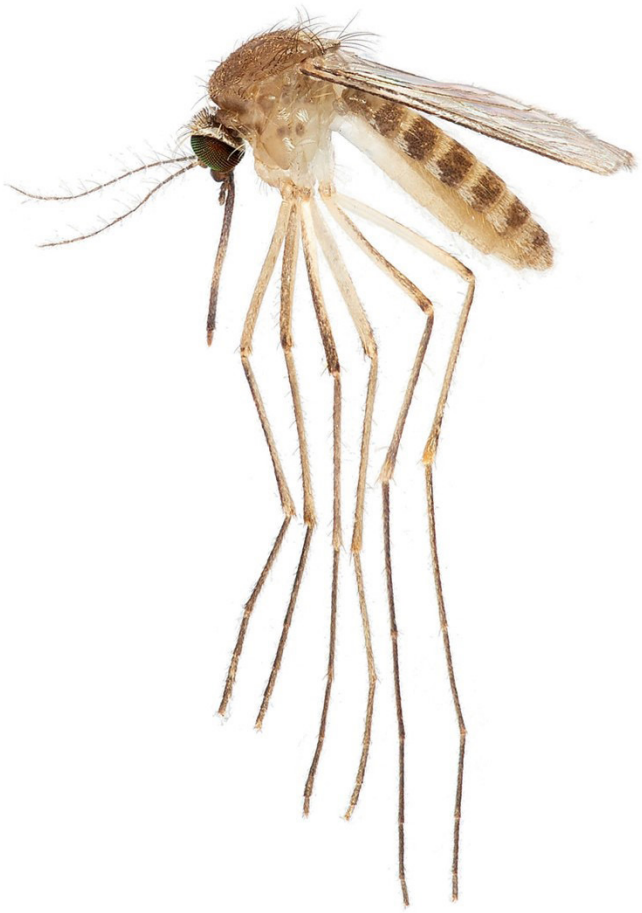

Figure 2. Lateral view of adult female *Culex interrogator* collected in Indian River County, Florida, 13 March 2019.

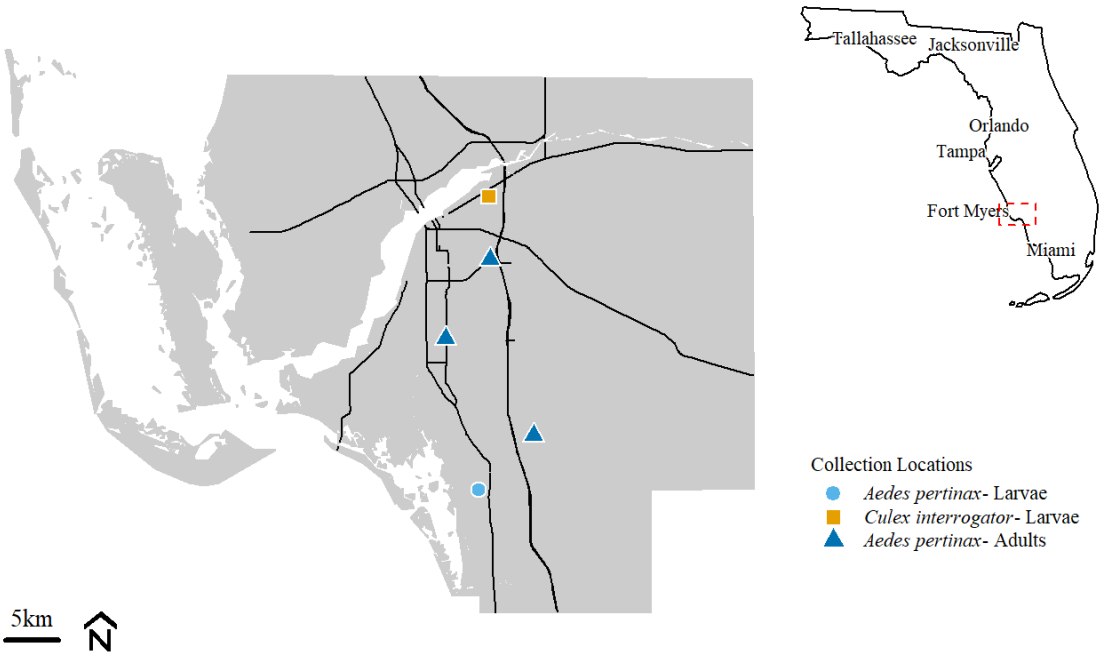

Figure 3. Map of Lee County displaying sites where *Aedes pertinax* and *Culex interrogator* were collected as larvae, or as adults in CDC light traps.

TWO NOVEL SINGLE NUCLEOTIDE POLYMORPHISMS IN THE VOLTAGE-GATED SODIUM CHANNEL GENE IDENTIFIED IN *AEDES AEGYPTI* MOSQUITOES FROM FLORIDA

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ABSTRACT

Aedes aegypti, the primary vector of dengue, Zika, chikungunya, and yellow fever viruses, is known to be resistant to pyrethroid-based insecticides in Florida. To improve our knowledge on the mechanism(s) responsible for this resistance, we sequenced 106 *Ae. aegypti* individuals collected from throughout Florida and examined mutations in a known insecticide resistance gene, voltage-gated sodium channel (VGSC; AAEL023266), also commonly known as the knockdown resistance (*kdr*) gene. Through whole genome sequencing, we identified 2 novel nonsynonymous single nucleotide polymorphisms (SNPs), F1174I and E478K, and 5 known SNPs, V410L, S723T, V1016I, D1763Y, and Q1853R, of which 4 were reported in Floridian *Ae. aegypti* for the first time. These SNPs provide a basis for further studies examining their contribution to pyrethroid resistant phenotypes, such as increased time of survival after insecticide exposure. This sequence data can be used to develop a multiplex genotyping assay to investigate the SNP frequencies in a larger number of samples and to examine their phenotypic contribution to pyrethroid resistance in *Ae. aegypti*.

Key Words: *Aedes aegypti*, SNP, Florida, resistance, *kdr*, pyrethroids

INTRODUCTION

Aedes aegypti (L). is found in peninsular Florida with no known established populations currently in the Panhandle (Parker et al. 2019). Where distributed in Florida, *Ae. aegypti* can be found in urban and suburban areas due to greater availability of artificial containers within these landscapes compared to rural areas (Braks et al. 2003). Urban landscaping additionally provides highly suitable larval habitats for production of several mosquito species. Ornamental bromeliads, which are commonly used for landscaping in tropical and subtropical areas, can also be utilized as a larval habitat by *Ae. aegypti* (Wilke et al. 2018, Brown et al. 2019). As the predominant mosquito species responsible for transmitting dengue, Zika, chikungunya, and yellow fever viruses, *Ae. aegypti* is an important public health vector. Currently, no vaccines are available for most *Aedes*-borne viruses, increasing the necessity to control mosquito populations to prevent local disease outbreaks.

While the best method for controlling mosquitoes is an integrated mosquito management plan that utilizes multiple techniques such as larviciding, biological control, and source reduction, adulticides are most frequently used by the 60+ mosquito control programs spread throughout Florida to reduce mosquito populations, especially during mosquito-borne virus outbreaks (Lloyd et al. 2018). Pyrethroids are among the most common insecticides utilized globally and within the state of Florida (Lloyd et al. 2018). These chemicals are synthetically derived versions of pyrethrins (Bond et al. 2014), naturally occurring insecticidal compounds found in the chrysanthemum flower, and are commonly utilized for mosquito control due to their characteristically low mammalian toxicity and broad-spectrum application (EPA 2009). Similar to DDT, pyrethroids bind to voltage-gated sodium channels (VGSC) affecting depolarization activity and leading to neuronal failure (Coats 1990). This class of chemicals is differentiated into two types (I and II) by the presence or absence of a α-cyano-3-phenoxybenzyl group (Coats 1990). Overuse of pyrethroids for controlling mosquitoes in addition to exposure to chemicals in urban runoff, used in agriculture and pest control, as well as from other sources can result in strong selection pressure towards resistant individuals. Pyrethroid resistance in Florida *Ae. aegypti* populations has been well documented (Estep et al. 2018, Parker et al. 2020, Schluep and Buckner 2021, Scott et al. 2021). In particular, Parker et al. 2020 recently tested 37 *Ae. aegypti* populations from across Florida and reported that 95% of these populations were resistant to at least one pyrethroid.

Genetic point mutations within the VGSC can confer pyrethroid resistance in mosquitoes. For example, some nonsynonymous point mutations, which cause changes in amino acid sequences, within the VGSC can adversely affect the ability of a pyrethroid to bind effectively to its protein channel, resulting in knockdown resistance (*kdr*) (Soderlund and Bloomquist 1990). Knockdown resistance has been documented in multiple mosquito species including *Ae. aegypti* (Brengues et al. 2003, Saavedra-Rodriguez et al. 2007, Reimer et al. 2008, Martins et al. 2009, Babu et al. 2015, Li et al. 2015, Mack et al. 2021). In *Ae. aegypti*, the 1016 and 1534 amino acid positions in the VGSC have become a focal point for determining pyrethroid resistance (Brengues et al. 2003, Smith et al. 2016). Adult *Ae. aegypti* mosquitoes with 1016 and/or 1534 mutation(s) have been shown to display increased insecticide resistance to pyrethroids (Ishak et al. 2015, Estep et al. 2018, Hayd et al. 2020). A recently studied VGSC nonsynonymous point mutation that results in knock down resistance is V1016I, an amino acid substitution at the 1016 position from a valine (V) to an isoleucine (I). *Aedes aegypti* from Florida exhibiting heterozygotic (V/I) and homozygotic (I/I) *kdr* genotypes at the 1016 position have been detailed in prior studies (Estep et al. 2018, Scott et al. 2021).

In addition to V1016I and F1534C, recent studies have identified three other single nucleotide polymorphisms (SNPs), V410L, S723T, and D1763Y within the VGSC that seem to be associated with pyrethroid resistance in *Ae. aegypti* (Haddi et al. 2017, Chung et al. 2019, Saavedra-Rodriguez et al. 2019). The V410L mutation was first identified in Brazilian *Ae. aegypti* populations (Haddi et al. 2017) but has also been recorded in *Ae. aegypti* populations in Africa and Mexico (Villanueva-Segura et al. 2020, Ayres et al. 2020). Recently, the D1763Y mutation was discovered in *Ae. aegypti* populations from Taiwan (Chung et al. 2019). In the continental United States, California experienced significant increased allele frequencies for the V410L, S723T, and V1016I mutations in two major cities between 2013 and 2018 and the detection of a new kdr muation, the Q1853R (Kelly et al. 2021). However, no studies have documented the occurrence of the V410L, S723T, D1763Y, and Q1853R mutations within *Ae. aegypti* from Florida.

Combinations of *kdr* mutations can also act synergistically with each other to increase pyrethroid insensitivity (Al Nazawi et al. 2017). Several studies have reported heightened levels of pyrethroid resistance in mosquitoes that express two *kdr* mutations compared to one (Du et al. 2013, Hirata et al. 2014, Al Nazawi et al. 2017, Haddi et al. 2017). For example, Haddi et al. (2017) witnessed higher deltamethrin and permethrin dose-response curves for *Ae. aegypti* with a V410L and F1534C profile compared to adults with the F1534C mutation alone. Additionally, Al Nazawi et al. (2017) reported increased time to mortality against deltamethrin in *Ae. aegypti* with a V1016G and S989P haplotype. In Florida, Estep et al. (2018) not only detected the V1016I mutation in *Ae. aegypti*, but also documented it co-occurring with the F1534C mutation. The F1534C mutation has the ability to confer pyrethroid resistance solitarily, however a stronger positive correlation between higher permethrin resistance ratios and higher homozygote resistant genotype, IICC, frequencies were observed for Floridian *Ae. aegypti* containing the mutations V1016I and F1534C (Estep et al. 2018). These three studies are important examples of the additive effect of *kdr* mutation combinations in increasing pyrethroid sensitivity.

As part of a larger project aimed at understanding dispersal of Floridian *Ae. aegypti* with respect to environmental conditions and/or landscape, we obtained 106 *Ae. aegypti* specimens from 15 counties in Florida between 2016-2021. Using whole genome sequence data, we screened for nonsynonymous SNPs occurring within the VGSC. Five previously documented point mutations (V410L, S723T, V1016I, D1763Y, and Q1853R) associated with pyrethroid-resistance and two novel point mutations (F174I and E478K) were identified. Here, we provide the geographic distribution, alternate allele frequency, and depth coverage of these nonsynonymous mutations.

MATERIALS AND METHODS

Mosquito Sample Collection

Between 2016-2021, interested Florida mosquito control programs were provided with an *Aedes* egg collection kit. The kit contained seed germination paper (Anchor Paper Express, Plymouth, MN) as an oviposition substrate, binder clips, 480 mL black plastic cups (Gary Austin Advertising, Jackson, TN), a microcentrifuge tube with 1:1 lactalbumin:yeast mixture as an oviposition attractant, and collection instructions (Parker et al. 2019). Oviposition cups containing seed germination paper were placed in the field by participants, and the seed germination paper was replaced once a week. Egg papers were collected and sent to the University of Florida, Institute of Food and Agricultural Sciences, Florida Medical Entomology Laboratory (UF/IFAS FMEL). Field-collected egg papers were air-dried, if necessary, then hatched in 1 L rearing trays. Larvae were fed 1:1 lactalbumin:yeast ad libitum. Pupae were transferred into water-filled cups and placed in a 30.5 x 30.5 x 30.5 cm Bug Dorm adult rearing cage (Bioquip©, Rancho Dominguez, CA). A cotton ball soaked in 10% sucrose solution was provided as a carbohydrate

source for emerged adults. All life stages of the mosquitoes are reared in a walk-in bioroom set at 83°F ± 2°F and 70% humidity ± 5% with a 12:12 LD photoperiod. Adults were identified to species by morphology (Burkett-Cadena 2013), and female *Ae. aegypti* mosquitoes were transferred to individual microcentrifuge tubes containing 70% ethanol solution for future DNA extraction.

DNA extraction/library prep

DNA was extracted from 106 individual mosquito specimens utilizing a magnetic bead-based DNA extraction protocol described by Chen et al. (2021). DNA concentrations were measured using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) and a Qubit instrument (Life Technologies, Carlsbad, CA) for each sample. A genomic DNA library was constructed with the QIAseq FX DNA Library UDI kit (Qiagen, Valencia, CA) using 20 ng input DNA for each mosquito. Enzymatic fragmentation was carried out at 32°C for 11 minutes followed by 65°C for 30 minutes. Ligation of adapters was performed at 20°C for 2 hours. PCR amplification of constructed libraries was conducted for 8 cycles of denaturation at 98°C for 20 seconds, annealing at 60°C for 30 seconds, and DNA extension at 72°C for 30 seconds. Library cleanup was conducted using PCRClean DX (Aline Biosciences, Woburn, MA). Library concentrations were measured with Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) and a Qubit instrument (Life Technologies, Carlsbad, CA).

Sequencing and data analysis

Constructed libraries were sequenced as 150bp paired-end reads using a NovaSeq instrument (Illumina) at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) Nextgen DNA Sequencing Core. Fastp version 0.20.1 was used to trim raw reads (Chen et al. 2018). Trimmed reads were mapped to the Ae13CLOV028MT (Genbank ID: MH348176) using BWA-MEM (Li 2013) version 0.7.15 recommended by Schmidt et al. (2018) to minimize the impact of mitochondrial reads mapping to the nuclear genome due to presence of pseudogenes (Hlaing et al. 2009). After mapping to mitogenome, unmapped and mate-is-unmapped reads were filtered utilizing Sambamba (Artem al. 2015), converted to fastq files using Samtools version 1.12 (Li et al. 2009) and mapped to the AaegL5 reference genome (Matthews et al. 2018) using BWA-MEM (Li 2013) version 0.7.15. Qualimap version 2.2 was used to calculate mapping statistics (Okonechnikov et al. 2016). Freebayes (Garrison and Marth 2012) version 1.0.1 with standard filters and population priors disabled was used for joint variant calling of all samples. Repeat regions were soft-masked in the AaegL5 reference genome and SNPs in these regions were removed from analysis. Further analysis only focused on biallelic SNPs with a minimum of 6X coverage. A 10% missing data threshold was used to filter SNPs.

Distribution maps of the SNPs were constructed using mapchart.net. A VGSC image was constructed using Protter version 1 (Omasits et al. 2014). Locations of SNPs were determined by alignment with *Ae. aegypti* (ACB37024.1) (*Aedes aegypti* genome working group 2017) and *Musca domestica* (ANW06229) (Scott et al. 2014) reference sequences transferring SNP annotations from *Aedes* to *Musca* to identify *Musca* protein position. Structural annotations were identified by alignment with *Drosophila melanogaster* (SCNA_DROME) reference sequence (Matthews et al. 2015).

We calculated the alternate allele frequency for each SNP identified by dividing the observed alleles for each genotype by the total number of copies of all the alleles at that particular genomic coordinate. Then the SNPs were classified as being common or rare in the *Ae. aegypti* sampled based on their calculated alternate allele frequencies. We considered any SNP identified in ⩾ 25% of *Ae. aegypti* as common and any SNP found in < 25% of *Ae. aegypti* as rare.

RESULTS

One hundred and six adult female *Ae. aegypti* mosquitoes were obtained from 15 counties in central and south Florida (Table 1). The genome of all 106 individual samples were successfully sequenced and filtered for nonsynonymous SNP mutations. Depth coverage for samples ranged between 7-10x approximately. In total, 7 SNPs within the VGSC were identified, 2 of which were novel (F174I and E478K) and 5 previously known (V410L, S723T, V1016I, D1763Y, and Q1853R).. Three of the four known SNPs (V410L, S723T, and V1016I) were observed in *Ae. aegypti* from all 15 sampled counties (Figure 1A), displayed alternate allele frequencies of approximately 70 to 73%, and were considered common (Table 2). The known SNP Q1853R was documented in *Ae. aegypti* from 11 of the 15 counties (over 70%; Figure 1B) sampled and was also classified as common due to its 26.7% alternate allele frequency. Interestingly, every individual that contained the Q1853R SNP also contained the V1016I mutation. We did not see a consistent co-occurrence between any other mutations. The final known SNP, D1763Y, was only found in two counties (Figure 1A) and displayed a minute alternate allele frequency of 1.0% (Table 2), which led us to classify this mutation as rare. The two novel SNPs identified, F174I

and E478K, were found in *Ae. aegypti* isolated to single counties and displayed alternate allele frequencies of approximately 1.0%, which also led to their classification as rare (Table 2). Structurally, the novel SNPs were located in the intercellular (E478K) and transmembrane (F174I), regions of the VGSC channel, respectively, based on constructed protein structure (Figure 2).

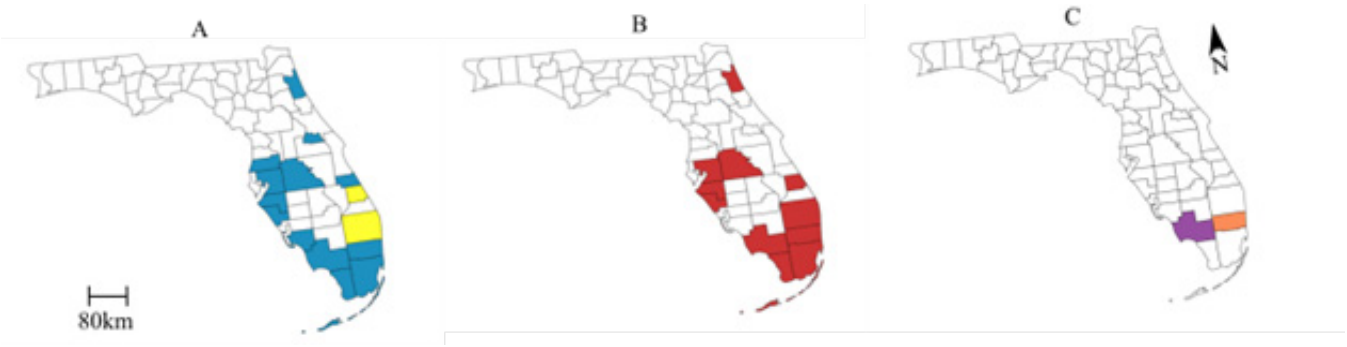

Figure 1. The geographic distribution of single nucleotide polymorphisms (SNPs) identified in *Ae. aegypti* mosquitoes collected from 15 counties in Florida. (A) Previously known SNPs, V1016I, S723T, and V410L, (blue), D1763, V1016I, S723T, and V410L (yellow); (B) Q1853R (red); and (C) the rare novel point mutations identified,, E478K (purple), and F174I (orange).

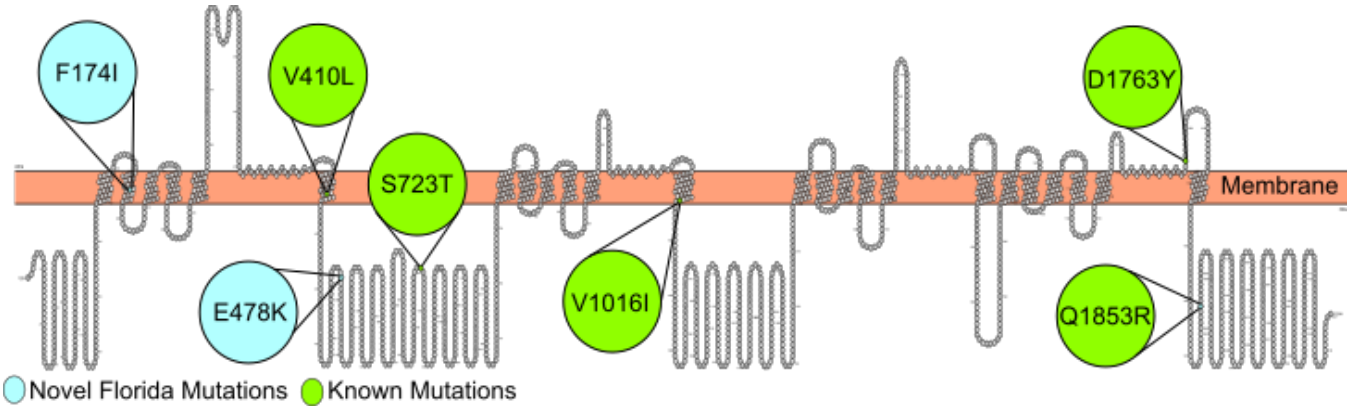

Figure 2. Topology of the mosquito sodium channel highlighting novel Florida and known SNP locations. Known mutation positions based on Mack et al. (2021). The VGSC is comprised of four homologous repeat domains (I-IV), each containing six helical transmembrane segments (1-6, 7-12, 13-28, 19-24 in Figure 2). Blue-filled circles indicate the two novel SNPs identified in this study. The green circles represent previously documented SNPs (Haddi et al. 2017, Mack et al. 2021).

Table 1. Collection sites of *Aedes aegypti* samples used in this study.

County	# of samples	City	Latitude	Longitude	CollectionYear
Broward	3	Davie	26.0693	-80.2082	2020
Broward	3	Hollywood	26.0350	-80.1768	2020
Broward	3	Miramar	25.9863	-80.2462	2020
Collier	9	Naples	26.1332	-81.7582	2020
Hillsborough	18	Tampa	27.9873	-82.4782	2020
Lee	2	Fort Myers	26.6528	-81.8118	2020
Miami-Dade	3	Miami	25.7546	-80.2235	2020
Monroe	4	Key Largo	25.0872	-80.4477	2020
Palm Beach	3	Haverhill	26.6886	-80.1135	2020
Pasco	3	Holiday	28.1863	-82.7452	2020
Pasco	2	New PortRichey	28.2619	-82.7055	2020
Polk	2	Auburndale	28.0497	-81.7767	2020
Polk	4	Haines City	28.1147	-81.6136	2020
Polk	3	Lakeland	28.1445	-82.0033	2020
Sarasota	6	Sarasota	27.3700	-82.4841	2020
Seminole	5	Sanford	28.8264	-81.3352	2020
St. Lucie	4	Port St. Lucie	27.3093	-80.3405	2020
St. Johns	4	St. Augustine	29.9012	-81.3126	2020
Indian River	3	Vero Beach	27.5872	-80.3734	2016
Manatee	24	Palmetto	27.5485	-82.5595	2018

Table 2. The positions of the single nucleotide polymorphisms with accompanied allele and amino acid information.

SNP Group	*Musca* aa[1] position	Chromosome	Genomic coordinate	Reference allele	Alternate allele	Alternate allele frequency	Aaeg[2] aa[1] position	Reference aa	Alternate aa	Mean Depth and STDV
Known	410	3	316080722	C	A	73.3%	408	V	L	7.63 +/- 4.34
Known	723	3	316014588	A	T	73.0%	711	S	T	8.65 +/- 6.10
Known	1016	3	315983763	C	T	70.4%	1012	V	I	7.47 +/- 3.61
Known	1763	3	315932009	C	A	1.0%	1794	D	Y	7.65 +/- 4.38
Known	1853	3	315931672	T	C	26.7%	1884	Q	R	9.67 +/- 7.32
Novel	478	3	316067895	C	T	1.1%	476	E	K	7.70 +/- 5.59
Novel	174	3	316101951	A	T	1.0%	189	F	I	8.23 +/- 4.69

[1]aa = amino acid
[2]Aaeg = *Aedes aegypti*

DISCUSSION

Aedes aegypti is an important vector species of dengue, Zika, and chikungunya viruses in Florida. The lack of developed vaccines for many of *Aedes*-borne diseases creates heavy reliance on mosquito population control for preventing disease transmission. Insecticidal use is an integral part of adult mosquito control in Florida (Lloyd et al. 2018). The presence and development of insecticide resistance among mosquito populations can potentially undermine the effectiveness of mosquito-borne disease control tools currently utilized.

However, studies like ours that identify and screen for SNPs in pyrethroid resistant populations could allow for detection of reduced insecticide sensitivity in mosquito populations, indicating a need for changes in control strategy. Our results corroborate Estep et al. (2018)'s findings of the V1016I mutation associated with pyrethroid resistance being common in Floridian *Ae. aegypti* mosquitoes. Additionally, this study is the first to document the occurrence and distribution of the V410L, S723T, D1763Y, and Q1853R mutations in Florida. Our findings also indicate a shared geographic distribution of two recently identified mutations, V410L and S723T, with the V1016I mutation in Florida. Two locations, Hollywood in Broward County and Haines City in Polk County, had only variant homozygotes (L/L, T/T, and I/I) for three of the previously documented mutations, V410L, S723T, and V1016I , in all sampled adult *Ae. aegypti* mosquitoes. Variant homozygotes for S723T and V1016I were additionally found in Davie in Broward County. All other sampled locations exhibited mixed genotypes of homozygote wildtype (V/V, S/S, and V/V) variant individuals and heterozygote individuals (V/L, S/T, and V/I).

While our study did detect the F1534C mutation previously reported in Florida *Ae. aegypti* by Estep et al. (2018), it was excluded from analyses, because its depth coverage was less than 6X. Upon further investigation, low mapping reads in the F1534C region on chromosome 3 were most likely responsible for the low depth coverage. The low mapping reads may have been potentially due to the presence of similar sequences on a portion of chromosome 1 and the F1534C region on chromosome 3 within the reference genome, which caused amplified segments to align to chromosome 1 instead of chromosome 3. This issue was not observed with the other detected mutations. Interestingly, Fan et al. (2020) did not detect the F1534C mutation in *Ae. aegypti* collected from St. Augustine, Florida. However, the authors did detect the F1534C mutation in *Ae. aegypti* collected from other locations around the world (Fan et al. 2020). Perhaps the different methodologies utilized in our study and by Estep et al. (2018) and Fan et al. (2020) may explain the differences observed regarding F1534C. Future studies using qRT-PCR and mutation-specific primers are planned to validate the detection of F1534C in our *Ae. aegypti* samples. The presence of Q1853R in over 70% of the counties sampled and high allele frequency (26.7%) suggest that this mutation arose much earlier in time, which allowed for further dispersal throughout the state. In addition the Q1853R mutation was also found co-occurring with the V1016I mutation in *Ae. aegypti* mosquitoes. This co-occurrence pattern is similar to V1016I + F1534C observations detected in *Ae. aegypti* by Estep et al. (2018). Whether Q1853R functions additively or multiplicatively with F1534C to affect pyrethroid resistance is yet to be determined. Further studies are needed to assess the impact of the co-occurrence of these mutations as well as the novel SNPs we detected on *Ae. aegypti*'s phenotypic response to insecticides.

The occurrence in only one county and low allele frequency (approximately 1.0%) documented for both of our novel SNPs, F174I and E478K, suggest that these mutations arose recently and have had a limited chance of dispersal to other locations. Still, it is plausible that these rare mutations could be detected in additional locations if sampling is increased to include a substantial number of individuals from other parts of the state. Additionally, larger scale population genetic studies involving increased sample sizes per city and/or county are needed to accurately assess the distribution of the novel mutations that we identified. A limitation of our study is the minimal sampling of approximately 2-24 mosquitoes per city. The resolution of allele frequency for any given city or county needs to be examined with much larger samples in future studies. Nonetheless, our genome data provides template sequences that future studies can use to develop genotyping assays to examine fine-scale abundance of SNPs identified and their contribution to phenotypic insecticide resistance.

The VGSC gene is one of hundreds of genes that have the potential to influence insecticide resistance (Saavedra-Rodriguez et al. 2008, Faucon et al. 2015, Campbell et al. 2019). As such, examining just the VGSC is only scratching the surface of potential functional changes in insecticide resistance genes. Our genome data will allow us to look for nonsynonymous mutations in other insecticide resistance genes. It is evident that Florida *Ae. aegypti* harbor many *kdr* mutations that can functionally impact insecticide resistance. In addition to the effort to characterize their functions in the laboratory, a multiplex SNP genotyping

assay will be needed to better characterize the geographical distribution of these VGSC mutations and monitor their spread.

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SEMI–FIELD EVALUATION OF ULTRA-LOW VOLUME (ULV) GROUND SPRAY OF AQUALUER® 20-20 AGAINST CAGED *AEDES ALBOPICTUS* AND NON-TARGET HONEY BEE, *APIS MELLIFERA*

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ABSTRACT

Application of permethrin products by ultra-low volume (ULV) spraying against the container-inhabiting mosquito *Aedes albopictus* (Skuse) has been used for many years, but the impact of the insecticides on domesticated honey bees, *Apis mellifera* (Linnaeus) is still lacking. The present study was carried out to evaluate the impact of the permethrin product, Aqualuer® 20-20 (active ingredient: 20.6% permethrin+20.6% Piperonyl butoxide) ULV sprays on caged *Ae. albopictus* and *A. mellifera* in open semi-field conditions with cages spaced at 3 m, 22.8 m, and 45.7 m downwind of the spray-truck path. The results indicated that ULV spray of Aqualuer 20-20 is highly effective against *Ae. albopictus* achieving 94% mortality at 22.8 m and 82% mortality up to 45.7 m downwind distance. The highest mortality of *A. mellifera* was only 72% at 3 m downwind distance, but the spray killed 42% of the exposed bees up to 45.7 m down the spray path. This semi-field study conducted during the day time indicates the high effectiveness of the ULV spray of permethrin against *Ae. albopictus* and its comparatively low impact on the direct exposed non-target honey bee, *A. mellifera*. Further studies designed to be conducted in the natural environment during its real-time operations following label instructions of the insecticide will help establish spraying guidelines to minimize any unfavorable impact on domesticated *A. mellifera* while having expected mortality effects on *Ae. albopictus*.

Key Words: *Aedes albopictus*, *A. mellifera*, ULV, integrated vector management, mortality

INTRODUCTION

Aedes albopictus (Skuse) is a nuisance and a potential disease vector of several emerging and re-emerging arboviral diseases (Paupy et al. 2009, Weaver & Reisen 2010). It has been shown to be capable of transmitting at least 26 arboviruses including dengue (Shroyer 1986, Reiskind et al. 2008, Thavara et al. 2009, Rezza 2012), chikungunya (Bonilauri et al. 2008, Vega-Rúa et al. 2014), Zika (McKenzieI et al. 2019) and yellow fever (Fadila et al. 2016, Amraoui et al. 2018) viruses in tropical and subtropical regions worldwide. With the emergence of Chikungunya virus (CHIKV) in 2006-2007 in many countries, *Ae. albopitus* has been implicated as a main vector of the disease (Gould et al. 2010). Since its discovery in Harris County, Texas, in 1985, *Ae. albopictus* has spread widely in the continental United States (Moore & Mitchell 1997). After the initial invasion of the northern parts in 1986 it has now well established across Florida (O'Meara et al. 1993, Hornby et al. 1994, O'Meara et al. 1995, Juliano et al. 2004, Reiskind & Lounibos 2021). Although low competence vectors for Zika virus, *Ae. albopictus* from Florida was found to be at least two times more susceptible to the infection than *Ae. albopictus* collected in Brazil, where an outbreak of Zika occurred in 2015 (Chouin-Carneiro et al. 2016). Control of the populations of *Ae. albopictus* has thus become a crucial component in any mosquito control program in Florida as well as across the world.

Chemical control using mosquito adulticides applied as ultra-low volume (ULV) sprays is often a key and effective component of integrated vector management (IVM) programs to reduce arbovirus vector and nuisance biting mosquitoes (CDC 2013, Faraji et al. 2016). The ultra-low-volume (ULV) space sprays spread small aerosol particles of insecticides targeting adult mosquitoes as they are flying (CDC 2003). Previous studies report on detrimental effects of direct exposure from both ground and aerial ULV insecticide sprays on non-target organisms like honey bees (Caron 1979, Pankiw and Jay 1992, Hester et al. 2001, Zhong et al. 2003). Some studies report ULV spraying of insecticides had little or no impact on other flying insects with medium to large body mass (Boyce et al. 2007, Kwan et al. 2009, Schleier III and Peterson 2010). In addition, Rinkevich et al. (2017) and Pokhrel et al. (2018) have demonstrated low impact of ground pyrethroid ULV sprays on honey bees while providing effective mosquito control.

In the past decade, the Anastasia Mosquito Control District (AMCD) of St. Johns County, Florida has conducted regular truck-mounted ULV sprays of the pyrethroid-

based Aqualuer® 20-20 (active ingredients-20.6% permethrin and piperonyl butoxide 20%, AllPro Inc., St. Joseph, MO) in response to residential complaints about nuisance problems caused by container-inhabiting mosquitoes. However, the effects of these ULV sprays on domesticated honey bees, *A. mellifera* the crucial pollinators for many economically important fruit crops, has not been evaluated. The domesticated honey bee provides greater economic benefit to people than any other arthropod found in Florida (University of Florida 2018) including the St. Johns county. Florida is also the nation's third-largest honey producer. The honey bees are crucial pollinators of many economically important fruit crops such as strawberries, blueberries, squash, watermelons and avocados. Furthermore, they support several other commercial activities including selling beeswax, pollen, royal jelly and propolis also known as "bee glue" (University of Florida 2018). Therefore, honey bee health is so important to Florida crop production. This preliminary study was carried out to determine the direct spatial impact of ULV ground-spraying of Aqualuer 20-20 under semi-field conditions on caged *Ae. albopictus* and domesticated honey bee, *Apis mellifera* (L.) so that ULV spraying guidelines could be improved accordingly.

MATERIALS AND METHODS

Ae. albopictus pupae were obtained from the United States Department of Agriculture-Center for Medical, Agricultural & Veterinary Entomology (USDA-CMAVE), Gainesville, Florida, and maintained in an insectary at 28±2°C, 40-70% relative humidity, and a photoperiod of 14L:10D until adult emergence. Adults were kept in flight cages in the same insectary and provided with 10% sucrose solution *ad libitum*. Adult females used in the semi-field trials were 4-7 days old. Female *A. mellifera* worker bees >7 d old were collected from frames of beehives from the honey bee apiary of the Entomology and Nematology Department of the University of Florida. Bees were collected and transferred to cages one day prior to the test. They were maintained in a laboratory with windows (natural photo period) at 22±2 °C with and an RH between 40-70%, provided with 50% sucrose solution *ad libitum*.

Semi-field evaluations (WHO 2009) of ULV application against the two species were conducted with in a 90 m x 90 m grid test site at the St. Augustine Gun Club, Florida. Ten female mosquitoes were aspirated into each of the 12 cylindrical screened paper cages (10 x 4 cm). Ten honey bees were introduced into each of 12 separate paper cages similar to the ones used for the mosquitoes. One cage with mosquitoes and one with honey bees were placed on each of 12 pipe stands at ~ 1.2 m above ground level in the field evaluation area. Three pipe stands with control cages were placed upwind of the spray zone, just prior to starting the treatment, and left in place for 15 min. After this period, the control cages were collected and returned to the laboratory to avoid exposure to pesticide applications.

Pipe stands for the treatment experiment were placed in a 3 x 3 grid with the three rows standing 3 m, 22.8 m, and 45.7 m downwind of the spray-truck path. A truck-mounted single-nozzle ULV cold aerosol sprayer (Guardian 95ES, ADAPCO, LLC, Sanford, FL) was driven at 16 kmph in a path perpendicular to the wind direction with a flow rate of 1.18-1.42 L/0.41 hectare and droplet size (mass median diameter) of 25.7 microns. The insecticide was diluted to 1:9 (Aqualuer 20-20:water) as per the label instructions. The treatment started 30.5 m prior to the first pipe stand of the row and was stopped 30.5 m after the last stand to ensure sufficient spray coverage. Cages were collected 15 minutes after the treatment and taken to the laboratory. Once collected, each mosquito and honey bee cage was provided with a cotton pad soaked in 10% sucrose solution (mosquitoes) or 50% sucrose solution (honey bees). The number of knocked-down mosquitoes and honey bees in each cage was recorded at 1 h and 12 h post-treatment. Mortality counts were taken at each 24 h and 48 h post exposure and percent mortalities were used in the analyses. Three successful replications were conducted between 07:30 h to 09:30 h with at least one week separating the evaluations.

SPSS (IBM SPSS Statistics for Windows, version XX (IBM Corp., Armonk, N.Y., USA) software package was used to analyze the arcsine transformed percent mortality data. Normality of data sets was determined by the Kolmogorov–Smirnov normality test. Means were compared using the independent t-test to determine the effect of the insecticide compared to controls. The differential effects of the insecticide at different upwind distances during different post-exposure periods were determined by using the two-way ANOVA test with post hoc Tukey pairwise comparisons. Significance level was maintained at 0.05.

RESULTS

ULV spray of Aqualuer 20-20 has induced a significant immediate (knockdown) effect followed by high mortality on *Ae. albopictus* and *A. mellifera* at both post exposure periods at all three downwind distances of the spray path, compared to respective controls ($P<0.05$ for all) (Table 1). The two-way ANOVA confirmed no interactions between the distance and post-exposure period on either knockdown or mortality of both species ($P>0.05$). As

observed by simple main effects, the effects on different post-exposure periods of knockdown and mortality were not significantly different in both species (P>0.05 for all). However, the downwind distance had a significant effect on both knockdown (F=3.453, P=0.04) and mortality (F=3.83, P=0.03) of *A. mellifera* only. The knockdown of *A. mellifera* was significantly higher at 22.8 m than 45.7 m (P=0.033) while the mortality was significantly higher at 3 m than 45.7 m (P=0.029). The highest knockdown of *A. mellifera* was at 22.8 m (91.1 ± 4.84) (mean ± standard error) while the highest knockdown of *Ae. albopictus* was observed at both 3 m and 22.8 m (84.4 ± 11.07) (Figure 1). With some recoveries of knocked-down *A. mellifera*, the highest mortality occurred at 3 m (72.2 ± 9.83) followed by 22.8 m (68.9 ±7.89) and 45.7 m (41.67 ±15.14) (Figure 2). Without any recovery, the highest mortality of *Ae. albopictus* was at 22.8 m (94.4 ± 4.44) and the lowest mortality was at 45.7 m (82.2 ± 10.9) with no significant differences between any pair of distances (P>0.05 for all).

As there were no significant interactions of distance and the species on both knockdown and mortality, the simple main effects of the two variables were observed. The effects of distance were significant on knockdown (F=3.552, P=0.032) as well as on mortality (F=3.452, P=0.036) of the two species. Although the effects of the two species on knockdowns were not different (P>0.05 for both), *Ae albopictus* had a significantly higher mortality (F=23.009, P<0.0005) than *A. mellifera* both at 22.8 m (t= 4.323, P<0.0005) and at 45.7 m (t=3.454, P=0.002).

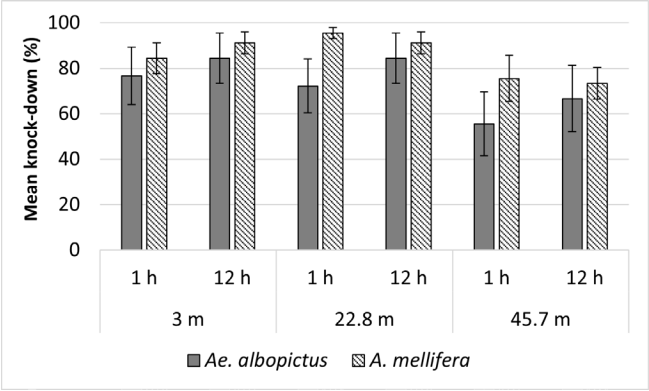

Figure 1. Mean percent knock-down of *Aedes albopictus* and *Apis mellifera* at different post-exposure periods of Aqualuer 20-20 at different downwind distances from the spray path (error bars indicate the standard error of the mean).

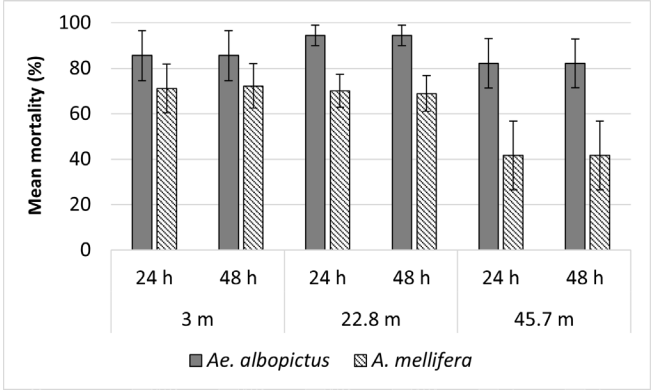

Figure 2. Mean percent mortality of *Aedes albopictus* and *Apis mellifera* at different post-exposure periods of Aqualuer 20-20 at different downwind distances from the spray path (error bars indicate the standard error of the mean).

Table 1. Effectiveness of ultra-low volume spray using Aqualuer 20-20 against *Aedes albopictus* and *Apis mellifera* compared to respective controls at different downwind distances and post-exposure periods (mean knock-down/mortality ± standard error of the mean).

	Aedes albopictus				*Apis mellifera*			
		Treatment				Treatment		
	Control	3 m	22.8 m	45.7 m	Control	3 m	22.8 m	45.7 m
1 h	1.11±1.11	76.7±12.7	72.2±11.9	55.6±14.1	0	84.4±6.7	95.6±2.4	75.6±10.2
12 h	1.11±1.11	84.4±11.1	84.4±11.1	66.7±14.7	1.11±1.11	85.6±11.2	91.1±4.8	73.3±6.9
24 h	3.33±2.36	85.6±10.9	94.4±4.4	82.2±10.9	2.22±2.22	71.1±10.7	70.0±7.3	41.7±15.1
48 h	3.33±2.36	85.6±10.9	94.4±4.4	82.2±10.9	5.56±3.77	72.2±9.8	68.9±7.9	41.7±15.1

DISCUSSION

This study was conducted to determine the effectiveness of ULV spray of Aqualuer 20-20 against *Ae. albopictus* and its impact on the non-target pollinator, *A. mellifera*. The semi-field experiments using caged insects exposed to the direct spray demonstrated its high effectiveness of 82 - 94% mortality against *Ae. albopictus* within 24 h, up at least to 45.7 m from spray-truck path, which is the maximum distance we tested. This result corroborates the findings of previous semi-field and field studies conducted with Aqualuer 20-20 and other pyrethroids (Farajollahi et al. 2012, Suman et al. 2012, Xue et al. 2013, Bengoa et al. 2014).

In contrast to the insignificantly higher knockdowns of *A. mellifera*, the effects of Aqualuer 20-20 ULV sprays were significantly higher on mortality of *Ae. albopictus*. This indicates a significant recovery of *A. mellifera* from immediate effects of the treatment which could be attributed to its larger body mass (Sanchez-Arroyo et al. 2019), as heavier insects exhibit decreased sensitivity to insecticides applied using ULV technique (Schleier and Peterson 2010a). Decrease in bee mortality with the increasing distance can be explained as a combined effect of the generally expected decrease in insecticide droplet concentration (Schleier and Peterson 2010b, Rinkevich et al. 2017) and the larger body mass of the bees. The highest mortality (72%) of direct exposed *A. mellifera* at 3 m downwind and 69% mortality at 22.8 m downwind are unlikely in operational conditions as many mosquito control programs create buffer zones around beehives. For example, AMCD applicators turn off sprayers at 30.5 m away from notified beehives. It should be noted that bee keepers across the county are encouraged to notify AMCD about locations of their beehives. The results of this semi-field, daytime experiment indicate that Aqualuer 20-20 ULV sprays could be used with high effects against *Ae. albopictus* at least up to 45.7 m while having ~50% less effects on *A. mellifera*. However, the noteworthy impact of 42% mortality of direct exposed *A. mellifera*, up to 45.7 m downwind distance should be further investigated in operational conditions. During operational mosquito control, ULV spraying is only limited to night and early morning applications when mosquito flight activity is high while honey bees are inside their hives and not active, thus not exposed directly to the air-borne insecticides. Therefore, carefully planned operational ULV spraying should have little opportunity to contact and kill honey bees while having a high impact on mosquitoes. Pokhrel et al. (2018) have demonstrated that operational ULV applications of different pyrethroid insecticides made just after sunset (between 7:00 pm to 10:00 pm) following label regulations and using properly calibrated equipment had no significant impact on *A. mellifera* in terms of mortality or brood development. Further studies under operational conditions and in the natural environment will help to look over these preliminary results obtained using caged insects that directly intercept insecticide droplets. Experiments that do not use forced exposure situations that are not normally encountered by bees would provide better insight on potential exposure hazards for bees. These results can help mosquito control programs interested, in taking extra precautions such as determining the buffer zone distance, time of spraying, when planning control operations in order to minimize the impact on honey bees.

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IMPACTS OF BARRIER INSECTICIDE MIXTURES ON MOSQUITO, *AEDES AEGYPTI* AND NON-TARGET HONEY BEE, *APIS MELLIFERA*

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ABSTRACT

Four novel commercial insecticide mixtures, composed of pyrethroid and nicotinoid active ingredients, were evaluated in a series of experiments in the laboratory, semi-field and field to determine acute toxicity (LC50) against pyrethroid-susceptible (ORL1952) and resistant (Puerto Rico) strains of *Aedes aegypti* L., and non-target adult European honey bees, *Apis mellifera* L. The four products were Tandem, Temprid FX, Transport Mikron, and Crossfire. The acute toxicity data showed that pyrethroid-resistant *Ae. aegypti* PR exhibited decreased sensitivity to all 4 insecticide mixtures, compared to pyrethroid-susceptible *Ae. aegypti* ORL1952. Tandem, Temprid FX, and Transport Mikron were more toxic to *Ae. aegypti* ORL1952 than to *A. mellifera*, but Crossfire was the least toxic. Transport Mikron was also more toxic to *Ae. aegypti* PR than to *A. mellifera*. The Honey bee Tolerance Indexes, determined with LC_{50} data of pyrethroid-susceptible mosquitoes, demonstrated that while Transport Mikron, Tandem, and Temprid FX were more toxic to *Ae. aegypti* ORL1952 than to *A. mellifera*, Crossfire was less toxic. The honey bee Tolerance Indexes decreased substantially when calculated with LC_{50} data from pyrethroid- resistant mosquitoes, but honey bees remained tolerant of Transport Mikron. Notably, while the insecticide mixtures did not control the PR resistant *Ae. aegypti* strain when applied as residual sprays to perimeter vegetation at label rates, susceptible *Ae. aegypti* ORL1951 were controlled, but applications affected honeybees (*A. mellifera*) for up to 28 days after treatment. Temprid FX resulted in 74% and 99% mortality, in adult *Ae. aegypti* ORL1952 and *A. mellifera*, respectively, for 28 days post-treatment. Transport Mikron and Tandem residues killed *Ae. aegypti* ORL1952 for up to 21 days post-treatment, while the effect of Crossfire lasted only 14 days. All three insecticides killed *A. mellifera* for up to 28 days post-treatment but at decreased mortality rates. For operational mosquito control, these data indicate that Transport Mikron has a reasonable safety margin (~25%) when targeting susceptible mosquitoes, compared to Tandem, Temprid FX, and Crossfire. The tested insecticide formulations need to be applied in higher doses to control resistant strains of mosquitoes that may be detrimental to honey bees. The ULV data indicated that pyrethroid resistance can be overcome with the insecticide mixtures.

Key Words: *Aedes aegypti*, *Apis mellifera*, insecticide mixture, non-target, barrier spraying

INTRODUCTION

Mosquito control programs aim to reduce mosquito-borne illness and nuisance mosquitoes through Integrated Mosquito Management (IMM) while limiting environmental impacts and preserving the integrity of non-target communities, which include economically and ecologically important populations such as honey bees and other pollinators (Sanchez-Arroyo et al. 2019 & 2021). Insecticide applications targeting adult mosquitoes are one of the major tools used in IMM. However, these types of broad-scale applications place mosquito control programs under public scrutiny with the public perception that adulticides contaminate the environment and have unintended impacts on beneficial insect populations. When mosquito control products are evaluated in laboratory and field settings, non-target impacts are often not evaluated and therefore, data on the effects of mosquito adulticides on non-target organisms is severely lacking, especially for honeybees (Qualls et al. 2010, Sanchez-Arroyo et al. 2019 & 2021, Giordano et al. 2020, McGregor et al. 2021). Although mosquito adulticide label restrictions and timing of applications aim to minimize impacts on non-target organisms, chemical exposure may occur through wind drift, plant contamination, and other unintended actions and uncontrollable factors.

In addition to the potential impacts of mosquito control insecticides on non-targets, the development of resistance in both nuisance and vector mosquito species to these insecticides is a global problem (Hemingway & Ranson 2000, Nauen R. 2001, Cui et al. 2006, Liu 2015). Thus, new commercial insecticides are needed for mosquito control programs. Recent studies evaluating insecticide formulations with multiple modes of action, mainly with

the combination of active ingredients for adult and larval control, have been demonstrated to be efficacious against resistant mosquito populations (Chung et al. 2001, Dantur et al. 2013, Jiang et al. 2017, Lei et al. 2019). Darriet & Chandre 2013 demonstrated that the combination of deltamethrin, piperonyl butoxide (PBO) and Group 1 neonicotinoids enhance control of resistant *Aedes aegypti* and *Anopheles gambiae.* By combining multiple modes of action, resistance mechanisms have been demonstrated to be overcome but little work has been done to evaluate the combination of multiple insecticide formulations and the impact this might have on non-target populations

Honey bees, in particular, are keystone pollinators in human agriculture and green spaces in urban and rural communities. Recently studies evaluating mixtures of biological and chemical insecticides (Chung et al. 2001, Luo et al. 2019) and/or larvicides and adulticides (Dantur et al. 2013, Darriet & Chandre 2013 ,Lucia & Harburguer 2009) with different modes of action have been reported against mosquitoes that demonstrate improved efficacy and a reduction in resistance. This study aimed to assess the impact of applications of mixtures of insecticides on mosquitoes and using the Western honeybee (*Apis mellifera*) as a model non-target organism, therefore providing mosquito control programs information on the selectivity of novel and registered insecticide mixtures. This information can guide mosquito control programs on operational control methods to minimize impacts on non-targets. Because the active ingredients proposed in this project have been assayed against *A. mellifera* as part of the registration process, it is expected that registered products will have a minimal effect on the bees.

MATERIALS AND METHODS

Mosquitoes. Two strains of *Ae. aegypti* were used in this study, the Orlando 1952 (ORL 1952) strain and the Puerto Rico (PR) strain which were obtained from the United State Agricultural Research Service Center for Medical and Veterinary Entomology in Gainesville, Florida and were maintained in colony at the Urban Entomology Laboratory at the University of Florida. *Aedes aegypti* eggs were added to trays containing 2.5 L of well water and maintained in an incubator at 28±2ºC, a 14 h light:10 h dark cycle and ~15 % RH until pupation. The developing larvae were fed with a food slurry consisting of 1: 1 brewer's yeast/ liver powder. Pupae were collected and maintained at 26±2ºC and 30 - 70% RH until adult mosquitoes emerged.

Honey Bees. Newly emerged *A. mellifera* adults and honey bee combs with capped brood (Figure 1a) were obtained from the honey bee Research and Extension Laboratory of the Entomology and Nematology Department of the University of Florida. The combs were kept at 33±2ºC, 25 - 30% RH and red light until adult bees emerged. One to three days post-emergence, adult bees were collected, and either used directly in the experiments or transferred to 'Bee Cups', and kept at 33±2ºC, 25 - 30% RH, and red light until assayed. The bee cups had ventilation holes and syringes filled with a 50% sucrose solution as food source for the bees (Figs. 1b,c).

Laboratory Evaluation. The insecticide mixtures used were Crossfire (MGK Insect Control Solutions), Tandem (Syngenta), Temprid FX (Bayer), and Transport Mikron (FMC) (Table 1). Tandem, Temprid FX, and Transport Mikron are registered for mosquito control while Crossfire is only registered for the control of bedbugs. All formulations are designed to be used as surface treatments and kill on contact and through residual activity.

Aqueous insecticide dilutions were applied uniformly to Whatman filter paper # 1 strips which were air-dried. Mosquito and honey bee bioassay strips had an area of 5 cm^2 and 14 cm^2, respectively. The mosquito and honey bee bioassay strips were treated with the same concentration (9 µL insecticide solution/cm^2).

Laboratory experiment. For the laboratory experiments, >3-day old adult susceptible and resistant female mosquitoes, *Ae. aegypti* were knocked down with CO_2, and the mosquitoes were transferred to 20-mL scintillation vials. Insecticide-treated paper strips were introduced to the scintillation vials after the mosquitoes had recovered completely from the knock-down. Ten females were used in three replicates of an insecticide concentration. Mosquitoes were fed with a 10% sucrose solution on a cotton ball for the duration of the experiments. *Aedes aegypti* mortality was assessed at 24±2 h.

Honeybees were knocked down with CO_2 and transferred to 4-ounce jelly jars. Insecticide-treated paper strips were introduced to the jars after the bees had recovered completely from the knock-down. Ten worker honey bees (3-10 d old) were exposed to each concentration of insecticides. *Apis mellifera* were fed with a 50% sucrose solution on a cotton ball for the duration of the experiment. *Apis mellifera* mortality was assessed at 48±2 h.

Experiment in greenhouses. The insecticide mixtures were diluted. based on the LC_{90}s generated in the acute toxicity studies and within range of typical Ultra Low Volume (ULV) applications. Tandem and Transport Mikron were diluted at a 1:8 ratio, while Temprid FX was diluted 1:56 and Crossfire was left undiluted.

For the ULV aerosol applications, aqueous insecticide mixture dilutions and water (negative control) were

Figure 1: a) Honey bee combs with capped brood and emerging bees. b) Bee cups with ventilation holes and syringes filled with 50% sucrose solution. c) Bee cups in 'Honey bee Hive Observation Room'.

Figure 2: Portable ULV Sprayer and Field Cages (Blue board was not present during application and was used only for better contrast in the picture).

applied to caged *Ae. aegypti*, ORL1952 and PR, and *A. mellifera* (from 3 beehives) with a Curtis Dyna-Fog Hurricane Ultra II electric portable aerosol applicator (Westfield, IN, ULV / mister) designed for spraying industrial and residential areas (Figure 2).

Droplet sizes of the different insecticide formulations were determined in triplicate with the Curtis Dyna-Fog Hurricane Ultra II electric portable aerosol applicator at the Anastasia Mosquito Control District, St. Augustine, Florida (AMCD) using an Artium Phase Doppler Interferometer (PDI), model TK1 (Artium Technologies, Sunnyvale, CA) which is capable of precisely measuring droplets from 0.7 - 150 µm (Table 2). The volume mean diameter (DV) $DV_{0.1}$, $_{0.5}$, and $_{0.9}$ represent the droplet size below which 10, 50 and 90% of the spray volume consists of droplets smaller than the listed size.

The insecticide trials were set up in a greenhouse located at the Entomology and Nematology Department of the University of Florida. The application rate was 1 oz (~30 ml) per 1000 cu ft which is typical for ULV aerosols and indoor use. Three replicates were conducted for each treatment and insect. Negative control treatments were set up before, between, and after insecticide treatments to check for ambient contamination with pesticides. The temperature inside the greenhouse ranged from 26-33°C during the experiments.

To cage the insects, mosquitoes and bees from three beehives, were first knocked down with CO_2. After mosquitoes had been immobilized, 10, 3-6 d old females were transferred to each treatment cage. After bees were immobilized, 10 newly emerged females from each bee hive were transferred to each treatment cage.

For each treatment with insecticide, the aerosol applicator was positioned 30 cm from the insects confined to field cages, which were attached to a wooden stake. The greenhouse ventilation was turned off during insecticide application. Fifteen minutes post-application, the greenhouse was evacuated of any residual insecticide mist for 15 minutes by turning on the ventilation remotely to avoid exposure of the operator to the pesticide application, after which the cages with treated insects were retrieved. Each treatment was set up in triplicates. A similar procedure was observed for negative controls where caged insects were treated with water rather than insecticide.

Treated cages with mosquitoes were kept at room temperature and ambient RH. Mosquitoes were fed with a 10% sucrose solution on a cotton ball for the duration of the experiments. Mortality was recorded immediately after treatment and at 24±2 h. Treated cages with bees were kept in the dark in a honey bee hive observation room maintained at 31±2°C and 15-30% RH. Honey bees were fed with a 50% sucrose solution on a cotton ball for the duration of the experiments. Mortality was recorded immediately after treatment and at 48±2 h.

Barrier Treatment Evaluation. For the barrier applications, aqueous insecticide dilutions were applied with Stihl *SR 450* backpack sprayers (Virginia Beach, VA) mounted on all-terrain vehicles. Applications were directed to perimeter vegetation and three potted azaleas (*Rhododendron* sp.) at the St. John's County Golf Course, St. Augustine, FL for each treatment (Tandem, Temprid FX, Transport Mikron, Crossfire, water = negative control). The insecticide mixtures were diluted to the high label rate concentrations for barrier applications (Table 3).

Potted azaleas were placed 30 m apart from each other within each treatment group, and the treatment groups were separated from each other by buffer zones of at least 304 m. The potted azaleas were not blooming at the time of treatment, but flowers developed 1-2 weeks after treatment. After treatment, potted azaleas were taken to Gainesville and placed outside at the Urban Entomology Building.

The residual effects of the insecticide mixtures were assessed on day 1, 7, 14, 21, and 28 post treatment using leaf bioassays on susceptible and resistant adult *Ae. aegypti* for the potted azaleas in Gainesville and on susceptible *Ae. aegypti* (ORL 1952) and *Culex quinquefasciatus* (Gainesville 1995 + Ocala 2003) for perimeter vegetation in St. Augustine.

For the experiments conducted at AMCD, two leaves adjacent to each other were collected from each plant and each time after treatment. The leaves were selected from the woody portion of the stems to ensure they were present when the plants were treated with insecticides. Two plastic Petri dishes were prepared for each plant and time after treatment: one for susceptible, and one for resistant mosquitoes. One leaf was placed into each dish with the treated side up. *Culex quinquefasciatus* and ORL 1952 strain were knocked down with CO_2, and 10, 3-6 d old females of each species were transferred into their own Petri dish. The Petri dishes were kept at room temperature and ambient RH. Mosquitos were fed with a 10% sucrose solution on a cotton ball for the duration of the experiments. Mortality was recorded at 24±2 h.

The residual effects of the insecticide mixtures on adult *A. mellifera* were assessed with azalea leaf bioassays on day 1, 7, 14, 21, and 28 post treatment. For the experiments, three souffle cups (one for each of three beehives used in the experiment) were prepared for each azalea and time point. Triplicates of five leaves were collected from each azalea and time point. The leaves were selected from the woody portion of the azalea stems to ensure they were present when the plants were treated with insecticides.

Table 1: Tested insecticide active ingredients and classes

Commercial Insecticide Name	Active Ingredient (A.I.)	A.I. - Class	A.I. (%)
Tandem	Thiamethoxam	Neonicotinoid	11.60
	λ-Cyhalothrin	Pyrethroid	3.50
Temprid FX	Imidacloprid [%],	Neonicotinoid	21.00
	β - Cyfluthrin	Pyrethroid	10.50
Transport Mikron	Acetamiprid	Neonicotinoid	5.00
	Bifenthrin	Pyrethroid	6.00
Crossfire	Clothianidin	Neonicotinoid	4.00
	Metofluthrin	Pyrethroid	0.10
	(Piperonyl Butoxide - synergist)	NA	10.00

Table 2: The volume mean diameter (DV) for diluted insecticides applied using a ULV sprayer is presented.

Product	DV 0.1 (x ± std. dev.) µm	DV 0.5 (x ± std. dev.) µm	DV 0.9 (x ± std. dev.) µm
CrossFire	34.2 ± 1.74 a	113.9 ± 1.08 a	140.9 ± 3.76 a
Temprid FX	18.7 ± 0.35b	39.3 ± 0.60 c	116.6 ± 10.11 bc
Tandem	18.2 ± 1.29 b	50.9 ± 4.20 b	131.5 ± 08.30 ab
Mikron	15.3 ± 0.78 c	34.0 ± 1.40 d	105.5 ± 9.06 c

*Means followed by the same letter within a column are not significantly different

Table 3: Amount of active ingredients applied in barrier trials

Insecticide	Dilution[a]	Percent A.I.[b] in Diluted liquid	Product (oz)/ 1000 sqft[c]	A.I.[d](oz)/ sqft	A.I. - Class
Tandem	1:115	Thiamethoxam (0.10) λ-Cyhalothrin (0.03)	2.2	0.347	Neonicotinoid Pyrethroid
Temprid FX	1:236	Imidacloprid (0.09) β – Cyfluthrin (0.04)	1.08	0.405	Neonicotinoid Pyrethroid
Transport Mikron	1:106	Acetamiprid (0.05) Bifenthrin (0.06)	2.4	0.291	Neonicotinoid Pyrethroid
Crossfire	1:9	Clothianidin (0.44) Metofluthrin (0.11) PBO[e] (1.11)	26	3.905	Neonicotinoid Pyrethroid Synergist

[a] Dilutions based on product labels
[b] A.I. = active ingredient
[c] All products applied at the rate of 2 gallons /100 sqft
[d] Combined a.i.s
[e] PB = Piperonyl Butoxide

Table 4. Acute toxicity of four insecticide mixtures for *Aedes aegypti* ORL1952 (pyrethroid-susceptible), *Aedes aegypti* PR (pyrethroid-resistant), and *Apis mellifera.*

Insecticide	*Aedes aegypti*		*Apis mellifera*
	ORL1952 - Susceptible	**PR - Resistant**	
	LC_{50} ± 95% Confidence Limits (µg/cm²) [a]		
Tandem	0.219 (0.131, 0.382) b	8.211 (0.168, 15.631) ab	1.723 (0.865, 2.655) b
Temprid	0.046 (0.023, 0.102) a	3.903 (1.548, 7.396) ab	0.300 (0.058, 0.653) a
Transport	0.128 (0.079, 0.206) ab	1.022 (0.466, 1.646) a	3.171 (1.481, 11.553) b
Crossfire	2.096 (1.731, 2.483) c	10.180 (5.379, 22.419) b	1.869 (1.055, 3.048) b
	LC_{90} ± 95% Confidence Limits (µg/cm²) [a]		
Tandem	0.663 (0.381, 4.753) a	43.522 (21.585, 16,467.304) a	4.240 (2.739, 12.965) a
Temprid	0.193 (0.092, 5.647) a	22.243 (10.461, 335.423) a	1.082 (0.549, 2273.330) a
Transport	0.341 (0.211, 1.391) a	3.270 (1.934, 23.425) a	12.506 (5.636, 17,421.779) a
Crossfire	2.934 (2.479, 4.781) a	29.416 (15.956, 718.725) a	3.820 (2.548, 34.958) a

[a]Of highest active ingredient (a.i.).
*Means followed by the same letter within a CL group for each species/strain are not significantly different

Table 5: *Aedes aegypti* insecticide resistance ratios and *Apis mellifera* tolerance ratios in relation to doses needed to kill *Aedes aegypti.*

Insecticide	Mosquito Index	Honey Bee Tolerance Index	
	Resistant/Susceptible LC_{50} Ratio[b]	**LC_{50} Ratio[d] to resistant *A. aegypti***	**LC_{50} Ratio[c] to susceptible *A. aegypti***
Tandem[e]	38.0	0.21	7.98
Temprid FX[e]	86.7	0.08	6.67
Transport Mikron[e]	8.1	3.10	25.17
Crossfire[e]	4.4	0.18	0.81

[a]Of highest active ingredient
[b]*Ae. aegypti* PR LC_{50} / *Ae. aegypti* ORL1952 LC_{50}
[c]*A. mellifera* LC_{50} / *Ae. aegypti* ORL1952 LC_{50}
[d]*A. mellifera* LC_{50} / *Ae. aegypti* PR LC_{50}
[e]Insecticide mixture (pyrethroid/nicotinoid)

Each set of leaves was placed into a souffle cup with the treated sides up. Ten newly emerged female honey bees from each of three hives were transferred to the souffle cup. Honey bees were fed with a 50% sucrose solution on a cotton ball for the duration of the experiments. The cups were kept in the dark in the honey bee hive observation room at 31±2°C and 15-30% RH. Mortality was recorded at 48±2 h.

Data Analysis. Data of the laboratory study were analyzed using generalized linear model procedures as implemented in SAS® PROC NLMIXED (SAS/STAT 15.1; SAS Institute, Cary NC) using a binomial distribution function and associated canonical logit link function. Because studies were repeated over time, time was considered a random effect. The fixed continuous effect was log10(rate). The LC50 was calculated as $-b_0/b_1$, where b_0 and b_1 are the intercept and rate parameter from the logistic regression model, respectively. LC50 was back-transformed to rate ± lower and upper 95% confidence limits and is reported as $\mu g/cm^2$.

Data from the field study were analyzed using generalized linear model procedures as implemented in SAS® PROC GLIMMIX (SAS/STAT 15.1; SAS Institute, Cary NC) using a binomial distribution function and associated canonical logit link function. For the mosquito study, fixed effects were insecticide, strain, day after treatment application (DAT), and all two- and three-way interactions. Replicate plot within each insecticide treatment was the sole random effects. For the honey bee portion of this study, fixed effects consisted of Insecticide, DAT and the Insecticide x DAT interaction. Because the honey bee response was based on replicated evaluations with bees collected from three hives, beehive was treated as a random effect in addition to replicate plots within insecticide.

RESULTS

Laboratory Evaluation. We determined the acute toxicity (LC_{50}) of Tandem, Temprid FX, Transport Mikron, and Crossfire for pyrethroid-susceptible and pyrethroid-resistant *Ae. aegypti* mosquitoes and *A. mellifera* honey bees. Based on these results, Honey bee Tolerance Indexes were calculated as the ratio of honey bee LC_{50} to mosquito LC_{50} (Table 4,5).

Higher concentrations of all four insecticide mixture formulations are needed to kill *Ae. aegypti* PR than *Ae. aegypti* ORL1952 (~38-fold more Tandem, ~87-fold more Temprid FX, ~8-fold more Transport Mikron and ~ 4-fold more Crossfire). The honey bee Tolerance Index decreased when calculated with *Ae. aegypti* PR LC_{50} rather than *Ae. aegypti* ORL1952 LC_{50} data, due to the high level of pesticide-resistance that has been observed in field populations of *Ae. aegypti*. Tandem, Temprid FX, and Transport Mikron were about 8, 7, and 25-fold more toxic to *Ae. aegypti* ORL1952 than to *A. mellifera* while Crossfire was less toxic (0.8-fold) to mosquitoes. The insecticide mixtures were all less toxic to *Ae. aegypti* PR than to *A. mellifera* at rates of ~ 0.2, 0.1, 3, and 0.2-fold (Table 5).

Greenhouse Evaluation. All insects died when treated with ULV sprays of Tandem, Temprid FX, Transport Mikron, and Crossfire at the rate of 1 oz (~30 ml) / 1000 cu ft. Difficulties encountered during these studies prevent conclusions to be drawn from this experiment.

Barrier Treatment Evaluation. Mortality at different time-points post treatment of susceptible and resistant mosquitoes and honey bees was determined after exposure to leaves of vegetation treated with Tandem, Temprid FX, Transport Mikron, and Crossfire at label rates for residual surface treatments (Table 6A, B). There was no *Ae. aegypti* PR mortality through exposure to the treated leaves with the sole exception of Temprid / day 1 (7% mortality). *Apis mellifera*, *Ae. aegypti* ORL1952, and *Cx. quinquefasciatus* were both affected by the insecticide residues left on the treated foliage, with high mortality (>75%) for 2-4 weeks with most products. Temprid had effective residual activity with 74% mortality for *Ae. aegypti* ORL1952 on day 28. The residual activity of Temprid also resulted in 99% mortality of *A. mellifera* up to day 28. Crossfire had the least effective residual activity and was the least toxic to both *A. mellifera* and *Ae. aegypti* ORL1952.

DISCUSSION

The research on target and non-target impacts of two-AI barrier insecticide mixtures for use in operational mosquito control was conducted to further understand the utility of novel combination insecticides for control of pyrethroid-resistant *Ae. aegypti* and the potential impacts on non-targets. The data shows that the barrier treatments with combination insecticides did not provide control against *Ae. aegypti* PR but they are effective for *Ae. aegypti* ORL1952. In addition, the dual-AI product Transport Mikron would be the best choice for controlling susceptible *Ae. aegypti* while minimizing non-target impacts.

Compared to acute toxicity data of commercial pyrethroid and organophosphate insecticide formulations by Sanchez-Arroyo et al. (2019), none of the insecticide mixtures tested in the present study had a lower LC_{50} for *Ae. aegypti* ORL1952 than Talstar or Mosquito Mist. The insecticide formulations tested by Sanchez-Arroyo et al. (2019) were Aqualuer (permethrin 20.6%, PBO 20.6%), Deltagard (deltamethrin 2.0%), Duet (prallethrin 1.0%

Table 6A: Mortality of *Ae. aegypti*, ORL1952 and PR, and *A. mellifera* exposed to treated leaves at different days after treatment (DAT) (Gainesville)

	Percent Mortality (95% Confidence Limits)				
DAT	NEG. CONTROL*	TANDEM*	TEMPRID FX*	TRANSPORT MIKRON*	CROSSFIRE*
			***Aedes aegypti* ORL1952 (n = 30 per DAT)**		
1	0 b	88 (71 - 95) b	99 (92,100) a	99 (95 - 100) a	07 (02 - 20) b
7	0 b	99 (92-100) a	99 (92 - 100) a	91 (78 - 97) b	0 b
14	16 (7-36) a	22 (9 - 44) c	96 (86 - 99) a	45 (23 - 69) c	23 (10 - 45) a
21	0 b	7 (02 - 19) d	73 (51 - 88) b	32 (15 - 57) c	0 b
28	0 b	0 d	74 (51 - 88) b	0 d	0 b
			***Aedes aegypti* PR (n = 30 per DAT)**		
1	0 a	0 a	9 (3 - 24) a	0 a	0 a
7	0 a	0 a	0 b	0 a	0 a
14	0 a	0 a	0 b	0 a	0 a
21	0 a	0 a	0 b	0 a	0 a
28	0 a	0 a	0 b	0 a	0 a
			***Apis mellifera* (n = 90 per DAT)**		
1	8 (3 - 20) a	98 (91 - 99) a	98 (91 - 99) a	87 (74 - 94) a	99 (94 - 100) a
7	7 (2 - 16) a	69 (50 - 83) b	99 (93 - 100) a	89 (76 - 95) a	26 (13 - 44) b
14	14 (6 - 29) a	72 (53 - 85) b	99 (93 - 100) a	60 (41 - 77) b	29 (15 - 47) b
21	13 (5 - 27) a	15 (7 - 30) c	99 (94 - 100) a	13 (6 - 26) c	12 (5 - 25) c
28	0 b	11 (5 - 24) c	99 (93 - 100) a	4 (1 - 12) d	3 (1 - 10) d

*Means followed by the same letter within a treatment group for each species/strain are not significantly different

Table 6B: Mortality of *Aedes aegypti* Orl1952 and *Culex quinquefasciatus* exposed to treated leaves at different days after treatment (DAT) (St. Augustine).

	Percent Mortality (95% Confidence Limits)				
DAT	NEG. CONTROL*	TANDEM*	TEMPRID FX*	TRANSPORT MIKRON*	CROSSFIRE*
			***Culex quinquefasciatus* (n = 30 per DAT)**		
0	0 b	67 (28 - 100) a	100 a	100 a	80 (41-100) a
7	0 b	80 (62-97) a	100 a	100 a	94 (76-100) a
14	0 a	63 (13 - 100) a	50 (0-100) a	61 (11-100) a	0 a
21	0 b	37 (0 - 78) ab	90 (48 - 100) a	74 (32 - 100) a	35 (6-77) ab
28	0 a	0 a	13 (0 - 35) a	17 (0 – 38) a	20 (0-41) a
			***Aedes aegypti* (n = 30 per DAT)**		
1	0 b	100 a	100 a	100 a	100 a
7	0 b	100 a	100 a	100 a	3 (0-6) b
14	0 (0 – 26) c	80 (54 – 100) ab	54 (28 -80) b	92 (65 – 100) a	0 c
21	0 (0-20) c	84 (64 -100) a	100 b	92 (72 - 100) a	57 (37 -77) c
28	0 a	46 (5 – 86) a	41 (0 – 82) a	41 (0 – 82) a	29 (11 - 70 a

*Means followed by the same letter within a treatment group for each species are not significantly different

+ Phenothrin 5.0%), Talstar (bifenthrin 7.9%), and Mosquito Mist (chlorpyrifos 24.6%). These were all single-AI products tested. Comparing the pesticides tested in the present study and those tested by Sanchez-Arroyo et al. (2019) demonstrates that Talstar and Mosquito Mist were the most toxic to *A. mellifera*. However, the dual- AI products tested in the present study, represent lower risk to honey bees than the single-active ingredient products tested previously (Sanchez-Arroyo et al. 2019), with exception of Temrpid FX. Transport Mikron, the insecticide mixture that contained the pyrethroid bifenthrin and the neonicotinoid acetamiprid, did not have a lower LC_{50} than Talstar, a bifenthrin insecticide, but its safety margin for *A. mellifera* was approximately 3 times higher than the safety margin of single-AI product, based on the honey bee Tolerance Index.

Overall, all of the insecticide formulations evaluated resulted in mortality to honey bees. For operational mosquito control in Florida, these data indicate that only Transport Mikron has a reasonable safety margin (~25%) when targeting susceptible mosquitoes, but Tandem, Temprid FX, and Crossfire should not be used. Thus, it is important to adhere to the restrictions stated on the pesticide labels to preserve honey bees and other non-targets. Most barrier application labels recommend applying the treatment to non-flowering vegetation to avoid non-target impacts. For the majority of mosquito control programs, the active ingredient (AIs) bifenthrin is the barrier treatment of choice and is one of the AIs in Transport Mikron. Since bifenthrin alone was demonstrated to be highly toxic to *A. mellifera* (Sanchez-Arroyo et al. (2019) but less toxic when combined with the neonicotinoid, acetamiprid, this combination may be more suitable for best management practices when using barrier applications for control of mosquito populations.

Another thing for mosquito control professionals to consider is when targeting insecticide-resistant mosquito populations, higher doses of the barrier products would be necessary. Given the non-target impacts described in the current study and Sanchez-Arroyo et al. 2019 and the fact that none of the products tested were sufficient at controlling the resistant PR strain, barrier applications in areas where resistant mosquito populations are documented would not be recommended and other methods such as ULV applications (Sanchez-Arroyo et al. 2019 and 2021) would be preferred.

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THE DOSE-PERSISTENCE RELATIONSHIP OF THREE TOPICAL REPELLENT COMPOUNDS AGAINST *AEDES ALBOPICTUS* AND *CULEX NIGRIPALPUS*

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ABSTRACT

The experimental piperidine compounds 1-(3-cyclohexen-1-ylcarbonyl)-2-methylpiperidine (AI3-37220), 1-(3-cyclohexen-1-ylcarbonyl) piperidine (AI3-35765), and *N, N*-diethyl -3- methylbenzamide (DEET) were evaluated for the persistence of repellency against laboratory-reared *Aedes albopictus* Skuse and *Culex nigripalpus* Theobald using a modified dose-persistence test procedure on human volunteers. The protection time (hours) provided by the tested repellent compounds against the two species of mosquitoes were proportional to the dose applied. Overall, higher application rates of each repellent compound were found to provide longer mean duration of protection from bites (MDPB) of the two species of mosquitoes. The repellent DEET tested against each mosquito species provided better protection time than the experimental repellent compounds AI3-37220 and AI3-35765. The three repellent compounds at 20 and 25% application rates provided the MDPB of *Ae. albopictus* for 5-8 hours and at 5, 10 and 15% provided the MDPB of *Cx. nigripalpus* for 5-7 hours. The MDPB provided by the three repellent compounds against *Cx. nigripalpus* was longer than that against *Ae. albopictus*. Also, the MDPB provided by each tested repellent compound varied from the individual human volunteer.

Key Words: *Aedes albopictus*, *Culex nigripalpus*, DEET, AI3-37220, AI3-35765, mosquito repellents

INTRODUCTION

Aedes albopictus Skuse and *Culex nigripalpus* Theobald are important vector mosquitoes of dengue fever and West Nile viruses (Shroyer 1986) and pests to humans and livestock (Shroyer 1986; Nayar 1982). The use of personal protective measures, such as the application of repellents, to reduce bites and risks of vector-borne diseases is especially important because of the environmental safety pressure of pesticide application and rapid or high resistance of mosquitoes to pesticides and pathogens to drugs.

Schreck and McGovern (1989) first reported that 25% of the novel piperidine compounds, AI3-37220 and AI3-35765 provided seven hours complete protection time, whereas 25% DEET as standard repellent provided more than eight hours protection time against *Ae. albopictus*. Barnard and Xue (2004) reported that DEET and three other repellents provided significant protection against mosquitoes from biting. More than a decade, AI3-37220 and AI3-35765 were evaluated against a wide variety of arthropod vectors and provided promising results in tests and demonstrated broad-spectrum arthropod activity (Coleman et al. 1993; 1994; Frances et al. 1996, Debboun et al. 1999). However, the dose-persistence relationship has not been directly determined in the standard mosquito repellent testing cage.

Buescher et al. (1982) used a small rectangular test cage with five circular openings and 15 mosquitoes to test the dose-persistence of DEET against *Ae. aegypti* (L.) on a human forearm. Coleman et al. (1993, 1994) evaluated the protection duration provided by the piperidine compounds AI3-37220 and AI3-35765 against some species of mosquitoes using the dose-response testing procedure on human subjects described by Buescher et al. (1982) and the American Society for Testing and Materials, E951-94 (ASTM, 1994). In this study, we directly used the mosquito repellent testing cages with 100 female mosquitoes, and exposure of the whole treated human forearm to detect the dose-persistence relationship of experimental repellent piperidine compounds AI3-37220, AI3-35765, and DEET against laboratory-reared *Ae. albopictus* and *Cx. nigripalpus*. This information could be used to assist in understanding the dose-persistence relationship and improve the skin repellent testing methods.

MATERIALS AND METHODS

Test Mosquitoes: *Aedes albopictus* mosquitoes were reared by the method described by Xue et al (1995). *Culex*

nigripalpus mosquitoes were introduced from University of Florida/IFAS, Florida Medical Entomology Laboratory, Vero Beach, Florida and reared by the method described by Nayar (1982) and modified by Barnard and Xue (2004). Blood meals for adult female mosquitoes of both species were obtained from restricted five to seven week-old chickens (Project A057 approved by the University of Florida, Institutional Animal Care and Use Committee).

Three hundred female *Ae. albopictus* mosquitoes were divided into three USDA's repellent testing cages (46 L x 38 W x 37 cm H) at 100 each and 300 female *Cx. nigripalpus* were similarly divided into three other repellent testing cages at 100 each. The six cages with two species of mosquitoes were divided into three groups at two cages each. One cage held *Ae. albopictus* and the other *Cx. nigripalpus* in each group. The biting pressure of mosquitoes in each cage was confirmed by exposure of a untreated forearm for 10 seconds before the conducting repellent testing. A 10% sugar cup was placed in each cage during the tests.

Test Repellent Compounds: The experimental repellent piperidine compounds, 1-(3-cyclohexen-1-ylcarbonyl)-2-methylpiperidine (AI3-37220) (98.5% liquid) and 1-(3-cyclohexen-1-ylcarbonyl)-piperidine (AI3-35765) (98.5% powder) were provided by Insect Chemical Ecology Research Laboratory, USDA/ARS, Washington, D.C. The repellent, N, N-diethyl-3-methyl benzamide (DEET) (95% liquid) was purchased from Virginia Chemical, Portsmouth, VA. Each repellent compound was diluted in ethanol from technical formulation to a series of concentration of 1, 5, 10, 15, 20, and 25%. The maximum concentration (25%) was selected based on our previous repellent testing results for the two species of mosquitoes in the laboratory (Barnard and Xue 2004).

Repellent Tests: One mL of each repellent concentration solution (in ethanol) was spread evenly on the forearm of a human volunteer, between the elbow and the wrist: area-covered = 648 cm^2 for volunteer one, 567 cm^2 for subject two, and 700 cm^2 for volunteer three. Extra amount of solution was running off from the skin in the smaller area. The same volunteers were used in all tests. Before starting each test, the volunteer used a vinyl glove to protect the untreated hands from mosquito bites. A total of three volunteers (IRB-01-445-96 approved by the University of Florida, Health Science Center, Institutional Review Board for Human Subjects) using six forearms participated in the experiments with two species of mosquitoes. The volunteers placed their arms into a repellent test cage for three minutes and observed mosquitoes that attempted to bite or bite on the exposed skin; removed their arms from the cage for a one minute rest period, followed by placing them in the next cage for three minutes, repeating the procedure until the mosquitoes in all cages were exposed to each treated arm. Tests were repeated for all six cages at 30-minute intervals. Each repellent concentration was repeated for three times by each subject for each species of mosquitoes. When more than one mosquito in a cage bite or attempted to bite during an observation period, the test for the dose and arm was ended and the mean duration of protection from mosquito bites (MDPB) calculated as the time between each repellent application and the multiple mosquito bites. If only one mosquito in a cage attempted to bite during an observation period, the treated arm was placed in that cage for 3 minutes after 30 minute interval. However, if no additional confirming bites were observed, testing of the cage was resumed until a confirmed bite was recorded. If there were no mosquito bites in a cage after 8.5 hours post treatment, the tests were stopped and the MDPB was recorded as 8.5 hours.

Design and Data Analysis: The experiments were run by factorial split-plot designs (Steel and Torrie 1980). Factor one consisted of three treatment repellent compounds (AI3-37220, AI3-35765, and DEET), factor two was six application concentrations (1, 5, 10, 15, 20, and 25%) of each repellent, factor three was two species of mosquitoes (*Ae. albopictus* and *Cx. nigripalpus*) for each concentration of the test repellents, and factor four was three human volunteers exposed to each concentration of the test repellents. A multi-way test was performed using the True Epistat computer program (Gustafson 1989). Raw data were transformed using the square root of (x+1) transformation to improve homoscedasticity before running the program. The relationship between the MDPB provided by each repellent and its concentration was separately conducted by the linear regression program.

RESULTS

The MDPB of *Ae. albopictus* provided by all DEET concentrations was greater than the experimental repellent compounds AI3-37220 and AI3-35765, whereas, the MDPB of *Cx. nigripalpus* was similar in all three repellent compounds (Table 1). The effect of three repellent compounds ($F=7.68$; df = 2,107, $P<0.001$), different concentrations ($F=105.15$; df =5, 107, $P<0.001$), and mosquito species ($F=164.88$; df =2, 107, $P<0.001$) were significant. The interactions of repellent compounds-doses ($F=2.17$, df = 10, 107, $P<0.05$), compounds-mosquito species ($F=10.69$, df = 2, 107, $P < 0.001$), and doses-mosquito species ($F=12.48$, df =5, 107, $P < 0.001$) were

Table 1. Mean duration of protection from bites (MDPB, hours ± SE) of *Aedes albopictus* and *Culex nigripalpus* on human forearm skin treated with six concentrations of AI3-37220, AI3-35765 and DEET.

	Mean duration of complete protection from bites					
	Ae. albopictus			*Cx. nigripalpus*		
Concentration (%)	AI3-37220	AI3-35765	DEET	AI3-37220	AI3-35765	DEET
1	0 ± 0	0 ± 0	0 ± 0	2.7 ± 1	1.7 ± 0.8	1.5 ± 0
5	0 ± 0	0.5 ± 0.4	3.5 ± 0.4	5.2 ± 1.3	5.2 ± 1.3	5.2 ± 0.6
10	1.8 ± 1.3	2.5 ± 1.1	4.0 ± 1.2	6.7 ± 1.3	5.8 ± 1.2	6.2 ± 1.7
15	3.8 ± 1.3	3.7 ± 0.6	5.7 ± 2.1	7.2 ± 0.9	7.2 ± 1.2	6.7 ± 1.3
20	5.3 ± 1.0	6.2 ± 0.9	7.2 ± 1.2	7.8 ± 0.5	7.2 ± 1.2	8.0 ± 0.4
25	7.2 ± 1.0	6.3 ± 0.2	8.0 ± 0.4	8.3 ± 0.2	7.5 ± 1.4	8.5 ± 0.0

F= 7.68, df = 2, 107, P <0.01

Table 2. Mean duration of protection from bites (MDPB, hours ± SE) of *Aedes albopictus* and *Culex nigripalpus* on three different human forearm skin treated with six concentrations of AI3-37220, AI3-35765 and DEET.

	Mean duration of complete protection from bites					
	Ae. albopictus			*Cx. nigripalpus*		
Volunteers	AI3-37220	AI3-35765	DEET	AI3-37220	AI3-35765	DEET
1	3.8 + 3.2	3.8 + 2.8	5.8 + 3.1	7.2 + 1.6	7.1 + 2.1	5.8 + 3.1
2	2.0 + 2.3	2.7 + 2.4	3.8 + 2.3	5.7 + 1.9	4.6 + 1.2	3.8 + 2.3
3	3.3 + 2.7	3.0 + 2.0	4.6 + 2.7	6.1 + 2.5	5.6 + 2.7	4.6 + 2.7

F = 4.338, df = 2, 53, P < 0.05

significant, too. However, the effect of the interaction of repellent compounds-doses-mosquito species was not significant (F=1.71; df = 10, 107, $P > 0.05$).

Both species of mosquitoes showed sensitivity to all repellent compounds and the MDPB provided by the repellent compounds had a relation to the concentrations applied. The linear relationships existed between the piperidine repellent compound AI3-37220 concentration and the MDPB of both species of mosquitoes, *Ae. albopictus* (r^2 =0.98; t= 18.426; df = 14; $P<0.01$) and *Cx. nigripalpus* (r^2 =0.86; t=6.30; df =14; $P < 0.01$). The similar linear relationships were found between the repellent compound AI3-35765 concentration and the MDPB of both species of mosquitoes, *Ae. albopictus* (r^2 =0.96; t=12.829; df =14; $P <0.01$) and *Cx. nigripalpus* (r^2 =0.76; t =4.375; df =14; $P <0.01$). In addition, the linear relationships were also found between the repellent DEET concentrations and the MDPB of the two species of mosquitoes, *Ae. albopictus* (r^2 =0.93; t =9.467; df =14; $P <0.01$) and *Cx. nigripalpus* (r^2 =0.85; t =6.037; df =14 ; $P <0.01$). Thus, the MDPB provided by the tested repellent compounds against the two species of mosquitoes were proportional to the doses applied. Logically, high dose of the repellents provided a longer protection time.

The MDPB of both species of mosquitoes provided by the three repellent compounds varied from the tested human volunteers (Table 2). The effect of human volunteers for the repellent compound tests was significant (F=4.338; df =2, 53; P =0.016), but the interactions of volunteers-mosquito species and volunteers- doses, and subjects-repellent compounds were not significant.

DISCUSSION

Results from this study showed that the two species of mosquitoes were easily repelled by the three different repellent compounds. More than 20% concentration of the three repellent compounds provided a satisfactory protection time for *Ae. albopictus* and more than 10% concentration of the three repellent compounds provided

satisfactory protection time against *Cx. nigripalpus* in the laboratory tests. DEET is the most effective repellent against *Ae. albopictus* and *Cx. nigripalpus*, compared to the two experimental piperidine repellent compounds AI3-37220 and AI3-35765.

Buescher et al. (1982, 1983) and Klun and Debboun (2000) developed a method and module to detect the optimal correlation between persistence and dosage of DEET applied on human skin. The similar methods for the sensitivity and dose-persistence of a variety of mosquito species to the novel piperidine repellent compounds AI3-37220 and AI3-35765 applied on human skin were used (Coleman et al. 1993, 1994). Currently, the method has been recommended as the American Society for Testing and Materials (ASTM) standard (1994). The apparatus and procedure consisted of a small testing cage with five 29 mm circular openings containing 15-20 mosquitoes and placed on a small area of the treated human forearm to test the dose-response and dose-persistence relationship each hour post treatment. However, this method may overestimate the repellent protection duration provided by tested repellents because it uses a small cage with small exposure area and small number of mosquitoes. In this study, we used a standard mosquito repellent testing cage containing 100 female mosquitoes per cage, and exposed an entire treated forearm for three minutes as a regular mosquito repellent test procedure. This method could reduce the influencing factors, such as small cage size, low mosquito density, and small exposure area.

The protection time of DEET against mosquitoes has been found to be influenced by different factors (Khan et al. 1975) and doses applied on human volunteers (Buescher et al. 1983). Higher doses applied in the repellent testing provided longer protection time against mosquito bites (Gilbert et al. 1955, Goodyer et al. 2020). The protection time provided by the two experimental piperidine repellent compounds against *Ae. albopictus* and *Cx. nigripalpus* related to the application rates and a high concentration of each repellent provided a longer protection time too.

Travis (1950) reported that repellent time varied greatly on different human volunteers treated by dimethyl phthalate (DMP) and against different species of mosquitoes (Rutledge et al. 1978, 1985). Low biting rates caused an extension of repellent time (Travis 1950; Barnard et al. 1998). In this study, our results showed that the MDPB provided by each repellent against the laboratory populations of *Ae. albopictus* and *Cx. nigripalpus* did not only vary from the tested species of mosquitoes, but also varied from the tested human volunteers.

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FIELD EVALUATION OF AUTOCIDAL GRAVID OVITRAPS AND IN2CARE TRAPS AGAINST *AEDES* MOSQUITOES IN SAINT AUGUSTINE, NORTHEASTERN FLORIDA

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ABSTRACT

Mosquito control programs are utilizing cost-effective long-term autocidal traps targeting the gravid population of container-inhabiting and other mosquito species, with the aim of reducing vector populations and disease transmission risk. In this field study we directly compared the efficacy of the Autocidal Gravid Ovitrap (AGO) and In2Care mosquito traps in St. Augustine, Florida. Total numbers of eggs (*Aedes aegypti* and *Ae. albopictus*) and adult mosquitoes were calculated at different weeks of trap deployment, pre-treatment (wk1-2), during-treatment (wk3-6), and post-treatment (wk7-8). There was a 72% reduction in both *Aedes* eggs in the two sites tested post-trap deployment, compared to pre-trap deployment. The mean numbers of eggs collected in the post-treatment, compared to pre-treatment showed that the In2Care traps had a higher reduction of mosquito oviposition (80%) than the AGO traps (23%). A total of 19 mosquito species included non container-inhabiting mosquitoes, *Aedes taeniorhynchus*, *Culex quinquefasciatus*, and *Cx. nigripalpus*, were collected by BG traps baited with BG lure and dry ice from the test sites. The species abundance varied between the two sites and collection weeks. The container-inhabiting mosquitoes, *Aedes aegypti* and *Ae. albopictus* were the major species. There was a significantly higher reduction in mosquito *Aedes aegypti* populations in the AGO (mean ± SE) (1.3 ± 1.7) and In2Care (4.9 ± 4.6) sites (X^2= 20.13, $P <$ 0.0001) post trap deployment, compared to pre-trap deployment. By week 8, the recovery rate of mosquito populations was highest in the In2Care trap site, followed by the AGO site. This result suggests that AGO traps were more effective than In2Care traps in reducing *Ae. aegypti* mosquito populations. For *Ae. albopictus*, the In2Care site had 100% reduction, and this was higher than the AGO site.

Key Words: *Aedes aegypti*, *Aedes albopictus*, Autocidal Gravid Ovitraps, In2care trap, Gravid mosquitoes

INTRODUCTION

Aedes aegypti (Linn.) and *Aedes albopictus* (Skuse) are highly specialized and selective domestic species that mostly oviposit in natural and man-made water containers associated with human dwellings and activities. Mosquito oviposition behavior (Bentley & Day 1989) has been a main target to develop novel approaches and tools for mosquito surveillance and monitoring vector population dynamics, and vector control (Reiter, 1983, Chadee and Corbet, 1987, Eiras et al. 2014). The first trap device used a combination of mechanical suction and organic plant-based infusion to collect eggs and attract gravid females (Reiter, 1983). Oviposition traps lined with polybutylene adhesive were successful to collect both *Ae. aegypti* and *Culex. quinquefasciatus* Say in Australia (Barbosa et al., 2010). This approach was further exploited and developed in attract-and-kill ovitraps and gravid traps, with the added advantage of attracting older mosquito cohorts that might be actively involved in disease transmission (Day, 2016).

The Autocidal Gravid Ovitrap (AGO) is a dual action surveillance and control tool that aims at capturing and killing gravid females of *Aedes* container-inhabiting mosquitoes (Barrera et al. 2014 a, b). The In2Care trap (In2Care) is a multi-purpose trap, containing both pyriproxyfen and the fungus *Beauveria bassiana.* Some field trials have been carried out to compare the efficacy of different trap types, such as gravid traps and AGOs under urban environmental conditions (Cilek et al. 2017) and AGOs and In2Cares (Buckner et al. 2017), where different levels of efficacies were observed (Su et al. 2020).

The AGO and In2Care traps have been preliminarily tested for control of *Aedes* mosquitoes in Saint Augustine, Florida (Autry et al. 2021). This is a continuation of direct comparison of the AGO and In2Care traps to determine their differential effectiveness against mosquitoes. Mosquito populations were monitored using host-seeking

Biogents-sentinel (BGs) traps (BioGents, Regensburg, Germany) and oviposition traps in both trap-treated sites. The expected outcomes of this study should inform mosquito abatement districts on the efficacy of the tested traps and the novel strategies for control of container-inhabiting mosquito vectors of diseases and nuisance species in urban areas.

MATERIALS AND METHODS

For this study, 100 AGOs and 100 In2Care traps were evaluated. Two sites were selected in downtown St. Augustine, Florida, based on their high abundance of *Ae. aegypti* and *Ae. albopictus* mosquitoes (Smith et al. 2018). All the traps were deployed over a one-day period, preceded by door-to-door interviews with residents of the households selected and providing educational brochures of the different traps being evaluated.

The selected sites were 18 acres (7.28 hectares) in size and 700 meters apart. Site 1 treated with AGOs and site 2 with In2Care traps. Site 1 had 91 houses, and Site 2 had 84 houses. Surveillance period (July 25-September 19, 2019) was 8-weeks and included pre-treatment for 2 wks, trap treatment for 4 wks and post-treatment (after trap removal) for 2 wks. Trap efficiency was carried out with nine ovitraps (1-L volume oviposition cups, ovicups) and six BGs traps. Three ovitraps and three BGs were deployed per site and remained throughout the whole 8-wk study period. The AGOs and In2Cares were used in the treatment period only. Ovitraps were fitted with seed germination papers and Cattail plant infusion water. The BGs traps were baited with BG-lure and dry ice.

The AGO trap was provided by SpringStar, USA. The trap consists of a 19-L black bucket with a fitted lid that houses a removable capture chamber. The capture chamber encloses a fitted sticky board and a small mesh screen on the bottom side of the capture chamber, which ensures the mosquitoes have no access to the water. Each AGO trap requires 8 L of water and no pheromones or pesticides are required. Holes were drilled at the 8-L mark to prevent excess water from rain or irrigation. The AGO traps were placed under trees, shrubs, and in the backyards to prevent damage or removal with 2-3 traps per household.

The In2Care trap, provided by Univar (Netherlands), is a small black bucket shaped like a planter pot. The trap lid has a 2.5 cm gap to the buckets rim that allows for mosquito entry but excludes debris and animals from the water inside the trap. Slots on the top of the trap drain excess water in the event of rainstorms and irrigation. This trap requires 3.5 L of clean tap water and a pre-supplied pesticide-treated gauze (includes the IGR, pyriproxyfen, the fungus *B. bassiana*, and Silicon Dioxide), which is placed onto a floating ring to keep the gauze upright. Two odor tablets supplied with the trap are added to the water to attract container-inhabiting mosquitoes. The In2Care traps were also placed under trees, shrubs, and in the backyards to prevent damage or removal with 2-3 traps per household.

The ovicups were black and could hold up to 750 mL of water and were purchased from Lowes, St. Augustine, FL. Each cup was filled with 500 mL of infusion water. To avoid overfill, a small hole was drilled above the water mark. Every week, the seed germination paper was collected, and new paper was placed with fresh infusion water.

A stock solution of infusion water was made from common Cattail plants (*Typha latifolia;* weighing around 1.36 kilograms; approximately 4-5 plants) collected from the field with green appearance. The Cattail plants were broken into smaller parts and placed in a large tank or dustbin and filled with water (up to 208.2 L mark) obtained from the retention pond onsite at Anastasia Mosquito Control District (AMCD), St. Augustine, FL. A stock solution of infusion water was prepared fresh at three-four days prior to putting the ovitraps in the field, to avoid over-fermentation and bacterial/mold growth. For effort and time effectiveness, infusion stocks were prepared for the whole experimental period and frozen and were thawed prior to field use.

Adult mosquitoes were collected from the BGs traps after 24 hr, while eggs were collected from ovicups weekly. The collected mosquitoes and egg papers were transferred to the AMCD lab for counting and identification of adult mosquito species.

All statistical analyses for AGO and In2Care trap data were analyzed using JMP statistical software. We explored the effects of AGOs and In2Care traps on *Ae. aegypti* and *Ae. albopictus* mosquito abundance and egg oviposition rates using a Shapiro-Wilk goodness-of-fit test along with a Kruskal-Wallis test, with significance levels set to 0.05. The data of non-targeted container-inhabiting species were not used and analyzed.

RESULTS

Mosquito species collected by BG traps. A total of 19 mosquito species were collected by BGs traps baited with BG lure and dry ice from the tested sites over the 8-wk period, with 18 and 17 species from Site 1 and Site 2, respectively (Table 1). The major species collected included target container-inhabiting mosquitoes *Aedes aegypti* and *Ae. albopictus*, and non container-inhabiting mosquitoes, *Ae. taeniorhynchus* Wied., *Cx. quinquefasciatus*

Table 1. Species of adult mosquitoes collected by BG sentinel traps baited with BG lure and dry ice from the AGO (site 1) and In2Care trap (site 2) on pre-treatment, during treatments, and post-treatments, St. Augustine, Florida, 2019.

Mosquito species	AGO	In2Care	All	Species % of Total
Aedes aegypti (Linn.)	**47**	**135**	**182**	**5.7**
Ae. albopictus (Skuse)	**758**	**96**	**854**	**26.8**
Ae. atlanticus Dyar & Knab	5	0	5	0.2
Ae. infirmatus Dyar & Knab	36	15	51	1.6
Ae. sollicitans (Walker)	0	4	4	0.1
Ae. taeniorhynchus (Wiedemann)	**55**	**646**	**701**	**22.0**
Anopheles Atropos Dyar & Knab	7	8	15	0.5
An. crucians Wiedemann	26	5	31	1.0
An. quadrimaculatus Say	11	2	13	0.4
Culex erraticus (Dyar & Knab)	30	1	31	1.0
Cx. nigripalpus Theobald	**263**	**143**	**406**	**12.8**
Cx. quinquefasciatus Say	**764**	**69**	**833**	**26.2**
Cx. restuans Theobald	2	1	3	0.1
Mansonia dyari Belkin, Heinemann & Page	2	4	6	0.2
Psorophora columbiae (Dyar & Knab)	11	5	16	0.5
Ps. ferox (von Humboldt)	4	2	6	0.2
Toxorhynchitus r. rutilus (Coquillett)	10	4	14	0.4
Uranotaenia sapphirine (Osten Sacken)	5	0	5	0.2
Wyomyia mitchelli (Theobald)	3	2	5	0.2
Total/Block	2039	1142	3181	100.0
(%) of total collected/Block	64.1	35.9	100.0	

Table 2. Number (mean ±SE) of target mosquitoes (eggs or adults) collected from different test sites, treated with AGO (Site 1) and In2Care (Site 2) traps on pre-treatment, treatment, and post-treatment, St. Augustine, FL, 2019. Different letters in column and row mean significant difference within the respective species.

Target	Traps deployed	Pre-treatment	Treatment	Post-treatment
Aedes aegypti & Ae. albopictus eggs	AGO	148.5±27.6 A	300.5±68.1 B	114.0±65.1 A
	In2Care	152.0±73.5 A	86.5±74.1 B	31.0±25.5 B
Aedes aegypti adults	AGO	1.0 ± 1.5 A	1.8 ± 2.0 B	0.83 ±1.2 A
	In2Care	3.8 ± 3.8 A	5.6 ± 5.3 B	4.7 ± 4.4 A
Aedes albopictus adults	AGO	19.2 ± 22.6 A	25.9 ± 21.4 A	17.7 ± 23.1 A
	In2Care	1.5 ± 2.8 A	4.5 ± 3.9 B	0.2 ± 0.4 A

Say, and *Cx. nigripalpus* Theobald, with variable abundance in different sites and collection weeks. *Aedes aegypti* and *Ae. albopictus* together represented 39.5% and 20.2% of total mosquitoes collected in Site 1 and Site 2, respectively.

Egg reduction of container-inhabiting mosquitoes. The total number of both *Aedes* mosquito eggs collected by ovitraps over the whole test period were the highest in the Site 1 (216±105 SE, n=1,727), followed by Site 2 (89±73, n=712). The mean egg numbers (eggs/week/trap ± SE) collected from post-treatment were significantly lower than those collected in pre-treatment and during the treatment (Table 2). Figure 1 shows that there is a general trend of reduction in eggs in the two tested sites based on the means in wk7 (1st week post-treatment) divided by the means in wk2 (2nd week pre-treatment); where the reduction rates were 59% and 88% in Site 1 and Site 2, respectively (Fig.1). Considering the whole period of post-treatment (mean of 2 weeks), compared to the pre-treatment (mean of 2 weeks), the reduction rates were 23% and 80% in Site 1 and Site 2, respectively. By comparing wk8 (2nd week post-treatment) to wk7, there were high recovery rates in mosquito populations, as indicated by increase in egg numbers by 130% and 300% in Site 1 and Site 2, respectively. The overall number of eggs collected in the two sites showed a 72% reduction in the post-treatment, compared to the pre-treatment period (Table 2 & Fig. 1).

Adult population reduction. Looking at the population dynamics of *Ae. aegypti* and *Ae. albopictus*, the two major dengue vectors targeted by the AGOs and In2Care traps, the results showed that in Site 1 and Site 2, the collected mean numbers of adult *Ae. aegypti* mosquitoes were 1.3 ±1.7 and 4.9 ±4.6, and *Ae. albopictus* were 22.2± 1.7 and 2.7± 3.6, respectively. In general, the two species *Ae. aegypti* and *Ae. albopictus* peaked at wk3 in the two treatment sites (Fig. 2 & 3). *Aedes aegypti* collections were higher in Site 2 than in Site 1 with mean (±SE) of 12.7±8.0 and 4.3±3.1 mosquitoes/trap, respectively. *Aedes albopictus* mean mosquito/trap (±SE) was highest in Site 1 (62.7±53.2) followed by Site 2 (14.0±12.1).

There was a reduction of 81.1% and 79.5% in *Ae. aegypti* mean numbers collected by BGs traps in wk7, compared to wk2 in both of Site 1 and Site 2, respectively (Fig. 2). By comparing mosquito mean numbers of wk8 to wk7, mosquito populations recovery rate was 353% in Site 2 higher than Site 1 with 85.7%. The mean numbers (i.e., mosquito/trap ±SE) in wk8 were 7.7±4.0 and 1.3±2.3, in Site 2 and Site 1, respectively (Fig. 2).

For *Ae. albopictus* collected by BGs, there was a general trend of population reduction by wk7, compared to wk2 in the two sites. The reduction rates were 100% in Site 2, compared to 72.8% in Site 1 (Fig. 3). By wk8, the mosquito

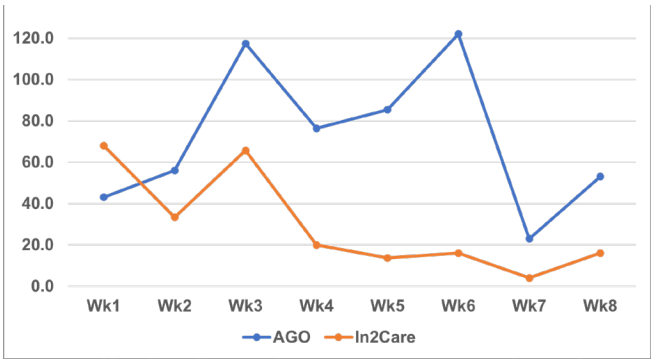

Fig. 1. Mean number of *Aedes aegypti* and *Aedes albopictus* mosquito eggs oviposited in ovitraps collected from different test sites, treated with AGO and (Site 1) and In2Care traps (Site 2) on pre-treatment, during treatment, and post-treatment.

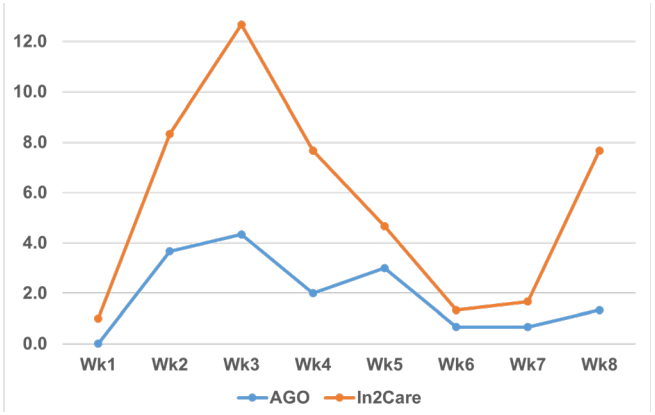

Fig. 2. Mean number of adult *Aedes aegypti* collected by BG sentinel traps from different sites, treated with AGO (Site 1) and In2Care (Site 2) traps on pre-treatment, during treatment, and post-treatment.

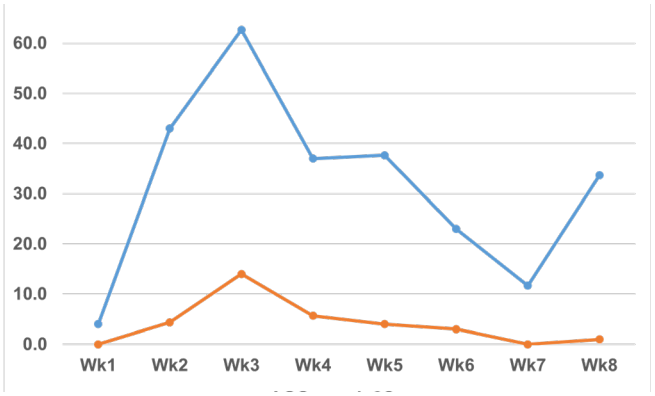

Fig. 3. Mean number of adult *Aedes albopictus* collected by BG sentinel traps from different test sites, treated with AGO (Site 1) and In2Care (Site 2) traps on pre-treatment, during treatment, and post-treatment.

population recovery rate was highest in Site 2 site followed by Site 1, with the means of 1.0±1.7 and 33.7±42.8 mosquito/trap in Site 2and Site 1, respectively.

For *Ae. aegypti*, in the treatment period, the mean mosquito numbers collected by BGs traps in Site 2 (5.6 ± 5.3 mosquito/trap) is 3-fold higher than in Site 1 (1.8 ± 2.0 mosquito/trap), but the difference is not significant (Table 2, Fig. 2). For *Ae. albopictus*, only in the treatment period, the mean mosquito numbers in Site 1 (25.9 ± 21.4 mosquito/trap) are significantly higher (X^2= 13.29, P = 0.0013) than Site 2 (4.5 ± 3.9 mosquito/trap) (Table 2 & Fig. 3).

DISCUSSION

In our study, we directly compared the field effectiveness of two new mosquito traps, the AGO and In2Care traps used as control tools, mainly against the major arboviral vectors and container-inhabiting, *Ae. aegypti* and *Ae. albopictus*.

Overall, the In2Care trap was the most effective in reducing mosquito populations for all container-inhabiting species collected at the end of the 4-wk trap deployment period, while the AGO was less effective. Looking at trap effectiveness on mosquito oviposition of *Ae. aegypti* and *Ae. albopictus*, via ovicups, the reduction rate observed in the In2Care site (88%) was higher than the AGO site (59%). However, in the post-deployment period, there was a remarkable increase in the total number of eggs in both In2Care and the AGO sites. This result shows a high recovery rate in the trap-treated sites, which could be considered an indication of the effectiveness of the In2Care and AGO traps. Ultimately, the In2Care traps had longer impact (i.e., after traps removal) on reducing the number of eggs laid by *Aedes* species than the AGOs (Fig. 1).

Due to the peculiar domestic container-inhabiting preference and oviposition behaviors of *Ae. aegypti* and *Ae. albopictus*, they have been the main species targeted for developing AGO and In2Care trapping strategies and tools, both for surveillance and control of these important arboviral vectors in different countries (Reiter, 1983, Ritchie et al. 2003,2009, Thavara et al. 2004, Gaugler et al. 2012, Barrera et al. 2014a,b, Buckner et al. 2017). In our study, the AGOs and In2Care traps had a significant impact on reducing adult *Ae. aegypti* populations, with the AGO traps being relatively more effective than the In2Care traps. Furthermore, after removal of traps, adult *Ae. aegypti* populations recovery rate in the AGO site was lower than in the In2Care site (Fig. 2). On the contrary, the In2Care trap were significantly more effective against *Ae. albopictus* adults than the AGO traps (Fig. 3). In Puerto Rico, AGO traps reduced *Ae. aegypti* populations by 60-80% with 85% area coverage (Barrera et al. 2014a,b). This reduction in vector population densities due to AGO deployment resulted in reduced transmission of Chikungunya virus (Barrera et al. 2016). Similarly, AGOs were effective in controlling gravid *Ae. aegypti* with good public acceptance in Australia (Mackay et al. 2013, Ritchie et al. 2009, Rapley et al. 2009).

The autodissemination stations (AS) showed variable efficacies against *Ae. aegypti* and *Ae. albopictus* in Florida based on mortality rates (29-45%) observed in sentinel ovicups, which measured the presence of competing natural oviposition sites and climatic conditions (Kartzinal et al. 2016). In addition, AS were able to transfer pyriproxyfen particles to most (85%) of cryptic ovicups and produced up to 41% mortality in *Ae. albopictus* pupal stages as well as a significant mortality in open ovicups. These mortality rates were compared to very low (0.3%) mortality in cryptic ovicups due to low-volume (LV)-*Bti* backpack sprayers use (Chandel et al. 2016). Similarly, in New Jersey, USA, Unlu et al. (2017) showed that pyriproxyfen-based AS were effective in reducing *Ae. albopictus* egg numbers and larval populations (collected by BGs traps, ovicups and sentinel cups), with significantly higher mortality in bioassays in trap-treated sites, compared to control sites. These studies are consistent with the partial efficacies of In2Care traps against *Ae. albopictus* in our study, which might be referred to the presence of cryptic or hidden larval sites, especially those created by the conditions of heavy rains such as from hurricane Dorian.

During this study, hurricane Dorian (August 29-September 5, 2019, i.e., wk6, the last week of trap deployment) caused heavy flooding, strong winds, abnormally high tides, and the destruction of environmental and artificial structures (roofs, trees, telephone poles, lawn décor, etc.) in both trap treatment sites. The intense wind and rain left debris in hard-to-reach areas as well as stacks of debris, which might have created new breeding sites and led to mosquito reinvasion into the treatment areas, especially for *Ae. aegypti* and *Ae. albopictus*. It is also possible that the intense wind and rain that came from multiple storms possibly flushed out the pyriproxyfen tainted containers in the In2Care traps resulting in pre-trap deployment-like conditions. The possibility for mosquito re-invasion into the treatment area is likely due to the surrounding housing and community structure in the treatment areas. However, the extent to which the homes and businesses surrounding both sites contributed to re-invasion is unknown.

A study using the In2Care trap showed that the combined use of IGR (pyriproxyfen) and entomopathogenic agents (the fungus *B. bassiana*) will

ultimately target all mosquito life cycle immature and adult stages (Snetselaar et al. 2014). In the first semi-field efficacy trials of In2Care trap as autodissemination stations-based intervention in Florida, USA, traps were effective in attracting gravid females of both *Ae. aegypti* and *Ae. albopictus* with significant inhibition of adult emergence from the traps (Buckner et al., 2017). Pyriproxyfen particles were successfully autodisseminated to new oviposition sites, which in turn resulted in significant reduction in newly emerged mosquitoes. There was also a significant reduction in adult survivorship due to water sites contamination with fungal spores. In an 8-12-wk field trial using pyriproxyfen- autodissemination station, there was moderate (50%) pupal mortality in *Ae. albopictus* in peri-domestic habitats and 50% and 40% mortality in junkyard and tire piles, respectively. Site contamination with autodisseminated pyriproxyfen particles was 82.2%, with detection of pyriproxyfen particles in sentinel cups at a long distance (200 m) from ADS installment areas (Suman et al. 2018).

These field studies show the differential effects of the AGO traps and In2Care traps on different *Aedes* mosquito species. Based on the trap efficacy data on *Ae. aegypti* and *Ae. albopictus*, the AGOs and In2Care traps can be deployed for up to five weeks in the field, with extended post-treatment period (e.g., 4 wks from the last trap deployment week) to span a complete mosquito gonotrophic cycle and in different mosquito seasons. In addition, the mosquito population dynamics should be assessed for each individual species. For broad assessment of effectiveness, the sticky papers in the AGOs can be used periodically to identify the range of mosquito species collected. Egg and larval stage surveillance will be useful to evaluate the latent effect of mechanical killing of adults by AGO sticky papers or due to insecticidal efficacy of IGR- and fungus-autodisseminated particles on mosquito populations, especially in cryptic or hidden larval water habitats or containers. This is an important factor to measure the potential and cost-effectiveness of AGO and In2Care traps against *Ae. aegypti* and *Ae. albopictus*. When feasible, the public health gains from deployment of these dual surveillance and control tools can be assessed based on the outcomes reflected on reduced disease cases or incidence in the trap deployment areas. The present study adds more field-based information on the AGO and In2Care traps as novel, cost-effective toolset, which can be used by mosquito control districts for IVM. However, additional investigations of mass-trapping and population monitoring schemes are needed to enhance their effectiveness in the field.

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STABILITY OF SENTINEL CHICKEN SERUM AT DIFFERING TEMPERATURES FOR WEST NILE VIRUS DETECTION

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ABSTRACT

The control of arboviruses is aided by surveillance programs. The use of sentinel chickens is a commonly used surveillance tool with operational benefits for mosquito control. However, sentinel chicken programs have associated costs related to animal husbandry, sample collection, and for out-sourced testing such as shipping costs. This study investigated the impact of eliminating cold shipping conditions often required for shipping samples to outside laboratories. Storage of sentinel chicken samples at room temperature (22°C) and 50°C for up to 6 days post blood draw and serum separation showed no difference in the reportable results with a commercially available competitive ELISA assay despite there being significant differences among some of the temperatures/days post blood draw. Eliminating the need for cold shipping conditions and the need for overnight shipping may reduce costs for mosquito control program.

Key Words: Costs, ELISA, chicken serum, stability, temperature, WNV

Mosquito-borne viruses continue to pose a threat to veterinary, wildlife, and public health on a global scale (Langevin et al 2001, Ramirez et al 2018). With the lack of preventative measures, such as vaccinations, and lack of treatment options, the control of arboviral activity is most effectively accomplished through the management of vector species, which can be enhanced through the use of surveillance measures (Ramirez et al 2018, Danforth et al 2021). Arboviral surveillance is thus a critical function to any effective mosquito control program (van den Hurk et al 2012). One commonly used surveillance tool is the use of sentinel chickens (Crans 1986, Olson et al 1991, Khan et al. 2017). Although the use of sentinel chickens is not always effective (Crans 1986, Day and Lewis 1992, Ramirez et al 2018), studies have shown how sentinel chickens can be one of the most sensitive indicators of virus activity in an area (Day and Lewis 1992, Reisen et al 1994, Ramirez et al 2018) and can lead to more precise data collection (Reisen et al 1994, Langevin et al 2001).

In Florida, sentinel chicken programs have been used since 1978 (Day and Lewis 1992) and are still used by mosquito control programs throughout the state today. Many programs rely on the data generated by these surveillance efforts to make informed decisions regarding their treatment initiatives (van den Hurk et al 2012). Sentinel chicken programs are also able to provide early warnings for human disease risk within communities (Day and Lewis 1992).

An established sentinel program does have inherent associated costs. In-house testing requires the cost of sentinel chicken husbandry, sample collection, and testing assays. Out-source testing requires the cost of sentinel chicken husbandry, sample collection, shipping of samples to outside laboratories, and if required, testing fees. Shipping costs regularly include the need for cold storage throughout sample collection and shipping (often accomplished through the use of dry ice or chilling packs; Reisen et al 1994) which also requires larger shipping containers – which increases costs as well. This study aimed to investigate the stability of sentinel chicken serum stored without the use of cooling agents as well as at higher temperatures that might be observed during the collection and shipping process.

Previously confirmed by the Florida Department of Health Laboratory, WNV-positive and WNV-negative sentinel chickens were bled using a 3-mL syringe and a 25-gauge needle with a brachial venipuncture (Johnson et al 2003, Florida Department of Health 2021). Blood was transferred into 3.5-mL serum separator tubes (SST) and allowed to clot for > 30 minutes. SSTs were then centrifuged at >2,000 revolutions per minute for at least 10 minutes (Grasedieck et al 2012). Serum was then removed from the SST and transferred into a 2-mL microcentrifuge tube (stock tube) that were pre-labeled with the individual bird IDs for storage. Stock tubes were stored in a temperature controlled incubator (22°C or 50°C) for the duration of sample collection. Subsamples (160 µL) were taken from the stock tubes and placed in a new 2-mL microcentrifuge tubes on days 0 (initial blood draw and serum separation), 1, 2, 3, 4, 5, and 6 post blood draw. After subsamples were collected these subsamples were immediately stored in a -80°C freezer until testing.

Sentinel chicken serum was tested for WNV antibodies using a commercially available competitive enzyme-linked

immunosorbent assay (ELISA) (Innovative Diagnostics, Grabels, France). In brief, controls and samples were mixed with dilution buffer and transferred into the pre-coated microwells of the ELISA plate. Plates were allowed to incubate for 90 minutes at room temperature and then washed three times (Model 1575 Immunowash Microplate Washer, Bio Rad, Hercules, CA) with approximately 300 µL of wash solution. Diluted conjugate was then added to each microwell and the plates were allowed to incubate for another 30 minutes at room temperature. Plates were washed again and substrate solution was added to all wells and allowed to incubate in the dark for 15 minutes at room temperature. Stop solution was then added to all wells and the plates were read at 450 nm (iMark Microplate Absorbance Reader, Bio Rad, Hercules, CA). Optical density (OD) values were then converted into a S/N% using the following equation: $((OD_{sample} / OD_{average\ negative\ control}) * 100)$. S/N% of >50 are considered negative, >40 but ⩽50 are considered doubtful, and ⩽40 are considered positive (ID.Vet 2020).

Subsamples from each day of collection were tested in triplicate. Comparison of sample averages between collection days was conducted using an ANOVA in Microsoft Excel (version 2016).

Sixteen blood draws were taken from 10 birds for analysis in this study (Table 1). OD values for specific samples and days were averaged and plotted on bar graphs. Samples P 1-4 and N 1-4 were held at 22°C for up to 6 days (Figure 1) and samples P 5-9 and N 5-7 were held at 50°C for up to 6 days (Figure 2). All results from each sample and their replicates were positive or negative as expected based on their pre-study status. Subsamples from day 6 of samples P 2 and N 1-2 were not collected as the stock serum had run out. Collection of subsamples from day 5 were missed for samples P 4 and N 4.

Significant differences were observed among the length of storage for samples P 1, P 4-8, N1-3, and N 5-7 (P 1: $F(6, 14)=5.18$, $p=0.005$; P 4: $F(5, 12)=3.46$, $p=0.036$; P 5: $F(6, 14)=3.26$, $p=0.032$; P 6: $F(6, 14)=10.22$, $p=0.0002$; P 7: $F(6, 14)=3.50$, $p=0.025$; P 8: $F(6, 14)=8.29$, $p=0.0006$; N 1: $F(5, 12)=9.23$, $p=0.0009$; N 2: $F(5, 12)=9.04$, $p=0.005$; N 3: $F(6, 14)=5.47$, $p=0.004$; N 5: $F(6, 14)=62.80$, $p<0.0005$; N 6: $F(6, 14)=125.61$, $p<0.0005$; N 7: $F(6, 14)=4.80$, $p=007$). No significant differences were observed among the length of storage for samples P 2-3, P 9, and N 4 (P 2: $F(5,12)=0.79$, $p=0.579$; P 3: $F(6, 14)=1.96$, $p=0.140$; P 9: $F(6, 14)=1.75$, $p=0.181$; N 4: $F(5, 12)=3.06$, $p=0.052$).

Other studies have investigated the integrity of serum and the detectability of a variety of components at different storage conditions and lengths of time. Grasedieck and colleagues (2012) demonstrated that there was no significant difference in the total RNA concentrations that were detected from serum after a year of storage at -80°C compared to storage at -20°C. Timms and colleagues (2007) determined that differing collection and handling methods influenced the protein profiles obtained from serum samples. Cruickshank-Quinn and colleagues (2018) observed differences in the metabolite abundance of serum based on time it takes to process samples.

This study demonstrated the stability of sentinel chicken serum kept above the chilled conditions which are typically used for storage and shipping. Despite there being significant differences observed between the S/N% among the collection days for a majority of the samples in this study, these differences are negligible as none of the differences effected the positivity or negativity of the respective samples (Figures 1 and 2). However, all negative samples had the highest S/N% (most negative value) on day 0 and then became more positive (lower S/N%) in the following days of storage. There was no real trend in which day experienced the "least negative" value. With this observation, there is the potential of a sample initially being negative, or in the doubtful range, on day 0 to then test positive after subsequent storage at higher temperatures. Unfortunately, it is difficult to experimentally show this potential as finding a sample that falls in or near the doubtful range is rare, and if found, often falls back into the negative range on a later blood draw (unpublished data). Conversely, some samples started trending back more negative by day 6. These results support the idea that storage of sentinel chicken serum at room temperatures or up to at least 50°C does not influence the detectability of WNV antibodies in competitive ELISA assays for up to at least 6 days post blood draw and serum separation.

Knowledge of the stability of sentinel chicken serum could be a considerable cost saving measure for mosquito control programs that ship their samples to outside laboratories with dry ice or other chilling measures. Also, the frequently required overnight shipping, which often has high associated cost, may no longer be require based on these results. As an example, if samples are collected on a Monday but the weekly receiving cutoff from the outside laboratory isn't until Wednesday, then there is no need to spend the extra funds to get your samples there on Tuesday. This is especially important for programs with limited budgets for arboviral surveillance (Peper et al. under review).

Knowledge of serum stability for sentinel chickens at higher temperatures is also beneficial to the operational side of mosquito control. If samples get delayed in shipment or left in the back of a field truck for a time, the results from this study help us understand the potential for these samples to still be of use for analysis. However,

Table 1. Bird identification number and associated Sample ID and West Nile virus status prior to sample collection and testing.

Corresponding Sample	Bird ID	Predetermined WNV Status
P 1–4	583	Positive
N 1–4	565	Negative
P 5	62	Positive
P 6	66	Positive
P 7	61	Positive
P 8	82	Positive
P 9	80	Positive
N 5	1080	Negative
N 6	1085	Negative
N 7	1086	Negative

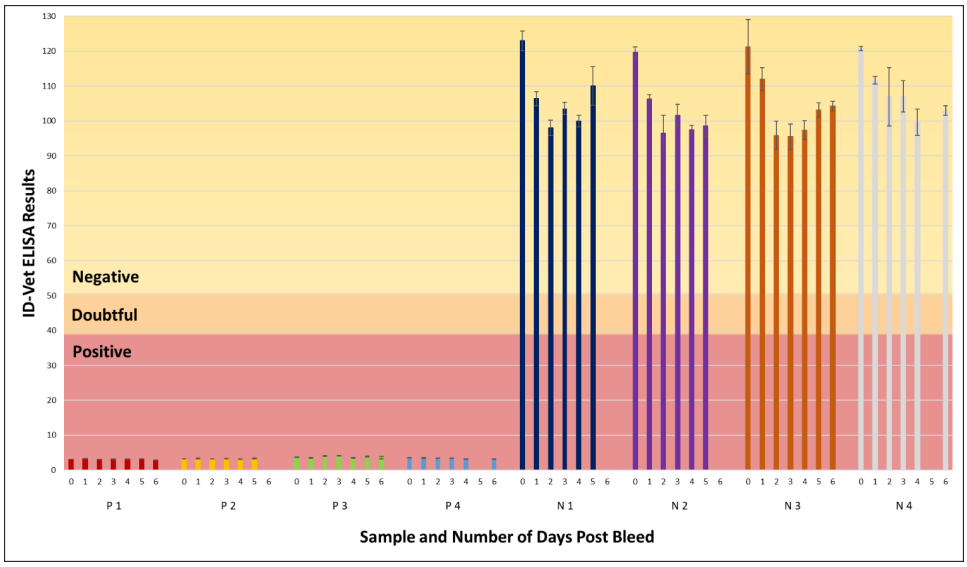

Figure 1. West Nile virus Competitive ELISA results for samples stored at 22°C.

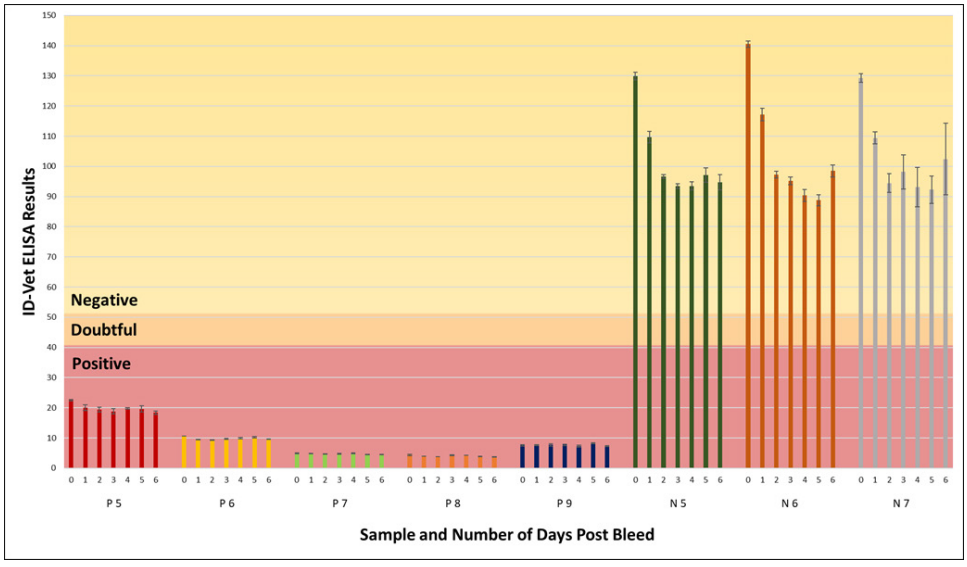

Figure 2. West Nile virus Competitive ELISA results for samples stored at 50°C.

some circumstances may expose samples to higher temperatures than used in this study. The elimination of dry ice in shipping would also reduce potential shipping hazards associated with the sublimation of carbon dioxide (Caldwell et al 2006) and packaging size of shipping container needed for transport.

The use of sentinel chickens for arboviral surveillance is a tried and true practice where the benefits often outweigh the negatives. One limitation, of course, for some mosquito control programs is the cost associated with shipping samples to outside laboratories for testing and this study helps lay the groundwork for updated sample handling protocols that may help reduce some of those associated costs.

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FIELD EVALUATION OF EMERGENCE TRAP DESIGN FOR MONITORING *MANSONIA* PRODUCTION FROM WATER LETTUCE *(PISTIA STRATIOTES)*

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ABSTRACT

Larvae of the mosquito species *Mansonia titillans* and *Mansonia dyari* attach to the roots of floating aquatic plants, primarily water lettuce (*Pistia stratiotes*) and water hyacinth (*Eichhornia crassipes*), to obtain oxygen and avoid predators. Surveillance for these species involves a robust monitoring program that identifies *Mansonia* habitat and production sites. This report evaluates floating emergence trap efficiency for *Mansonia* surveillance and identification of production sites. Three trap designs were utilized in the evaluation trials, including standard passive emergence traps, modified (active) emergence traps containing a CDC-light trap with and without standard incandescent bulbs. Overall, the active emergence trap with light resulted in the collection of a significantly higher number of emerging mosquitoes.

Key Words: *Mansonia*, emergence trap, water lettuce, water hyacinth

There are approximately 25 species of the genus *Mansonia* Blanchard known throughout the world, two of which are found in the state of Florida (Rojas-Araya et al. 2020). *Mansonia dyari* (Belkin, Heinemann and Page) and *Mansonia titillans* (Walker) larvae procure oxygen and avoid predation by attaching their siphon to the roots of floating aquatic plants, specifically, water lettuce (*Pistia stratiotes* L.) and water hyacinth (*Eichhornia crassipes* (Mart) Solms), respectively (Slaff and Haefner, 1985). This unique behavior renders traditional surveillance methods difficult. These species are fierce biters, most active during sunset, and travel 1300 meters on average from their emergent habitat (Verdonschot and Besse-Lototskaya 2014). *Mansonia* species are potential vectors for filarial nematodes, including dog heartworm (*Dirofilaria immitis* (Leidy)) (Bemrick and Sandholm 1966) and lymphatic filariasis (*Wuchereria bancrofti* (Cobbold)) (Ughasi et al. 2012). In addition, there have been instances where wild-caught females were found to be infected with West Nile virus (Unlu et al. 2010), St. Louis encephalitis virus (Beranek et al. 2018), and Venezuelan equine encephalitis virus (Sudia et al. 1971).

Controlling *Mansonia* involves a strong integrated approach by reduction of the aquatic host plants, as well as targeting the immature and adult stages of the mosquitoes (Rojas-Araya et al. 2021). Vector control agencies that focus on *Mansonia* control should adopt a monitoring program that identifies *Mansonia* habitat and production sites. Because of their intimate relationship with host plants, identifying *Mansonia* production sites and surveying for larval distribution can prove difficult (Service, 1993). Plans for the control of *Mansonia* species can be based off the trapping of newly emerged adults using emergence traps, providing a measure of mosquito production and an estimation of emergence from a given habitat.

Until recently, there was no aquatic plant or *Mansonia* production site monitoring program in place by the Collier Mosquito Control District (CMCD), located in Collier County Florida. In an effort to investigate whether the presence of water lettuce significantly contributes to CMCD's mosquito abundance, we began developing a monitoring program for *Mansonia* larval habitat, adult production, and adult abundance. Sampling adult emergence using the typical passive emergence trapping methods proved difficult, with small capture rates due to a combination of predation, length of time spent in the emergence chamber, and loss of emerged mosquitoes from catch container transfer. Due to CMCD's large-scale monitoring program, aspiration of adult mosquitoes from emergence traps to increase capture efficiency was not feasible as the process can be time consuming and labor intensive. Based on the above considerations, new sampling tools capable of overcoming the known constraints in *Mansonia* monitoring are continually needed. This study aimed to compare capture efficiency of the original pyramidal passive emergence trap design (Slaff et al, 1984) to two modified active emergence traps.

Three trap designs were utilized in the evaluation trials, including passive emergence traps (Figure 1A),

modified (active) emergence traps containing a CDC miniature light trap (CDC-light trap) (John W. Hock Company, Gainesville, Florida, USA) baited with and without a standard incandescent bulb (Figure 1B). Traps were built in-house using PVC pipe, mesh screen and foam pool floats. The PVC pipe was used to construct a 0.37 m^2 (4 ft^2) base and pyramidal structure reaching a height of 0.5 m (1.64 ft). Foam pool floats were attached to the base for floatation and mesh screen comprised the walls. Weatherproof ammunition boxes holding 6-volt batteries were used to power the CDC-light traps. To avoid battery flooding caused by rapidly rising water and trap placement, 0.37 m^2 (4 ft^2) floating platforms were designed to keep the batteries above water. The floating platforms were constructed of a rectangular PVC pipe frame, mesh screen to increase surface area, and foam pool floats (Figure 1B). For passive traps, a funnel was placed atop the apex of the pyramid structure with a modified catch container (Figure 1A), which consisted of a 1.5-qt plastic funnel directed to a 2 qt plastic container and lid outfitted with a 10.16 cm (4 in.) plastic circular louver and wire mesh for ventilation. Active traps were designed for CDC-light traps to be seated with a catch container hanging down inside the trap enclosure. The incandescent bulbs were removed from three CDC-light traps for use in the active emergence trap without light. No carbon dioxide or any other attractants were used.

The area used for the trap evaluation trials was routinely mapped using a DJI Mavic Pro Platinum unmanned aerial vehicle (SZ DJI Technology Co., Ltd., Nanshan, Shenzhen, China) to create orthomosaic maps, computationally inspect habitat, and pinpoint trap placement through the Drone Deploy mapping software (Drone Deploy Inc., San Francisco, California, USA) (Figure 1C). Traps were placed at 9 locations at least 3 m apart along the perimeter of the pond located in Ave Maria, Florida, containing full surface coverage of water lettuce (Figure 1C). The type of trap placed at each location was determined by creating a blocked randomization list using a pseudo-random number generator (Sealed Envelop Ltd., 2021). The evaluation was conducted over three weeks in April 2021, with three trap periods each lasting a duration of four days. The randomization list for trap type placement was regenerated for each trap duration.

Trap collections were brought back to the laboratory after each four-day trap duration. Insects collected from the traps were cold anesthetized and adult mosquitoes were identified by morphology (Burkett-Cadena 2013). Consistent with previous emergence-trap data collected by CMCD (data not shown), *Ma. dyari*, *Anopheles crucians* complex (*An. crucians* (Weidermann), *An. bradleyi* (King)), *Culex nigripalpus* (Theobald), and *Uranotaenia sapphirina* (Osten Sacken) were the most prevalent species collected (Figure 2A). Passive emergence traps and active emergence traps without light consistently produced fewer mosquitoes per trap duration with 2.61 ± 2.55 and 4.11 ± 3.01 (Mean ± SD) mosquitoes collected in total on average, respectively (Figure 2A). Both trap designs captured *Ma. dyari*, *Anopheles crucians* complex and *Cx. nigripalpus*, while *Ur. sapphirina* was not detected in either the passive or active-without-light trap designs. The combination of light and fan (active with light) facilitated the largest number of mosquitoes caught per trap duration, collecting a total of 23.94 ± 6.68 mosquitoes on average (Figure 2A). The active emergence trap with light collected a significantly higher number of mosquitoes than the passive emergence trap ($t = 5.17$, $df = 4$, $p = 0.006$) and the active emergence trap without light ($t = 4.69$, $df = 4$, $p = 0.009$). Interestingly, *Ur. sapphirina* was only collected in active emergence trap with light. There were no significant differences in catch rates by week.

Using our emergence-trap data, we estimated the relative production of *Mansonia* mosquitoes per acre of water lettuce with each trap design for the trapping duration. Production per acre was estimated by using the number of mosquitoes produced in 0.37 m^2 and converting to number of mosquitoes produced in 1 acre. Estimates from data obtained through the passive emergence trap and active emergence trap without light were low, with 20,570 ± 20,641 and 9,680 ± 5,834 (Mean ± SD) *Mansonia* mosquitoes per acre, respectively. The active emergence trap with light estimated significantly more *Mansonia* mosquitoes per acre with 173,029 ± 51,889 compared to estimations determined using the passive emergence trap ($t = 4.73$, $df = 4$, $p = 0.009$) and active emergence trap without light ($t = 5.42$, $df = 4$, $p = 0.006$).

Active emergence traps with light were the most reliable trap design evaluated in this study, catching the highest number and a more diverse collection of emergent mosquitoes. In addition, *Ur. sapphirina* were only found in the active emergence trap with light. Due to placement of traps and identical emergence trap bases, the total number of mosquitoes and species emerging should not have been impacted by the presence or absence of a trap light or fan. The light of the active emergence trap likely attracts the emerged mosquitoes to the fan, therefore catching more adults than the passive emergence trap or active emergence trap without light. Using the mounted CDC-light trap on an emergence trap base eliminates labor-intensive and time-consuming collection methods. In previous passive trap surveys, freshly emerged adult *Culex* mosquitoes spent ~1 day in the emergence enclosure before reaching

Figure 1: Emergence trap design and placement for evaluation. (A) Passive emergence trap design. (B) Modified (active) emergence traps containing a CDC miniature light trap (CDC-light trap), and (C) Orthomosaic map depicting the 9 trap locations.

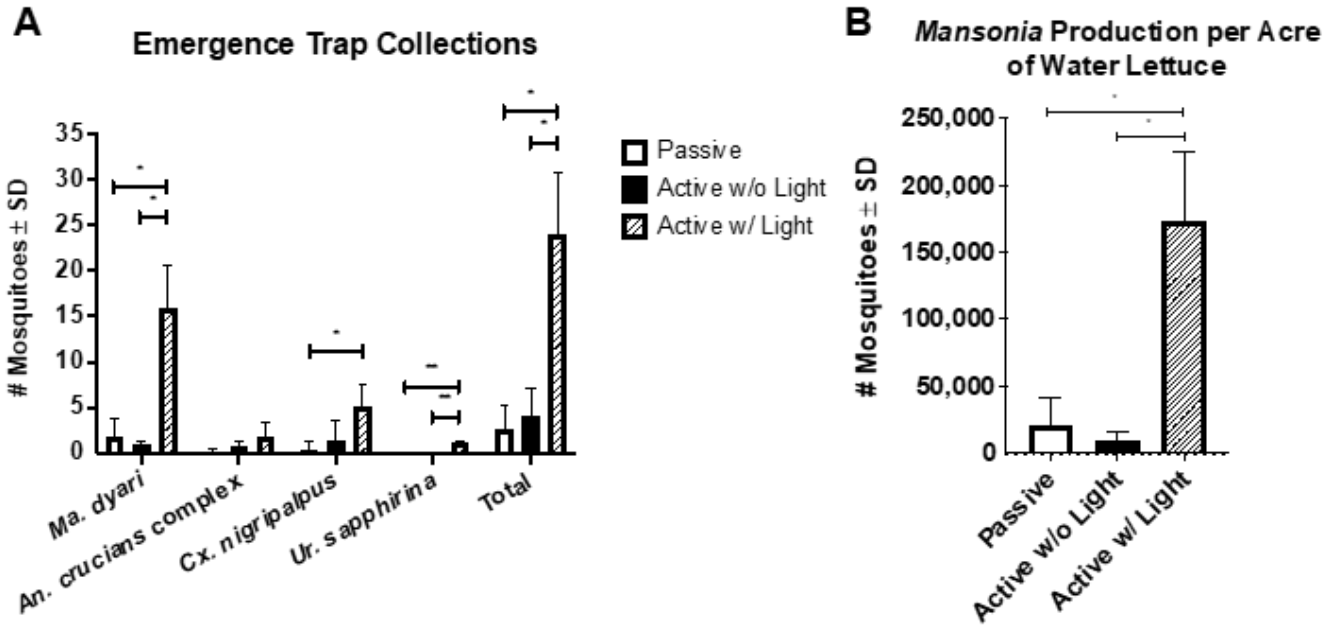

Figure 2: Trap collections and estimates of production. (A) Average number of mosquitoes collected in the three emergence trap designs for all three trap durations. (B) Estimates of *Mansonia* production per acre of water lettuce by trap type. Data is represented as Mean SD, * denotes $p < 0.05$.

the catch container (Walton, 2009). However, our studies indicate that the passive trap design results in extensive loss of emerged mosquitoes and reduced attractiveness to *Uranotaenia* species. It is important to note that the light emitted by the CDC-light trap may create a sampling bias by attracting more larvae under the floating emergence trap. Larvae of several mosquito species have been shown to be attracted to aquatic light traps (Service et al. 1983, Beehler & Webb 1992, Hribar & Hribar 2006). Further research is required to understand the attractiveness of the above-water incandescent light of the active emergence trap for *Mansonia dyari* larvae and if this results in an increase in catch rates.

In addition, the construction and utilization of the floating battery platforms further increase our emergence trap efficiency when running emergence traps on a 4-day period. During our rainy season (July-October), water levels at trap sites can drastically change in that time causing the battery to flood without the battery platform and leaving gaps in our surveillance data. The floating platforms allow movement with the flux of water level and lessen strain on the wires. The addition of the floating platforms also allows for overall better trap placement. In several surveillance sites, water lettuce is present beyond other submergent aquatic plants several meters off the bank. Previously, trap placement was limited by the length of wires from battery to the trap. Now traps have been repositioned to areas fully covered by water lettuce.

Overall, our active emergence trap design in combination with floating battery platforms has enhanced our efficiency in collecting mosquito species associated with aquatic plants. Through this trapping method, CMCD has been able to map and define mosquito species derived from aquatic weed habitat. This monitoring program has allowed CMCD to define the scope and need of an aquatic weed control program in Collier County. By identifying production sites and estimating *Mansonia dyari* production per acre of water lettuce, we can now target these areas for mechanical removal or herbicide application of aquatic weeds or for larvicide application for immature stages of *Mansonia* species.

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BEHAVIORAL RESPONSE OF ADULT *ANOPHELES QUADRIMACULATUS* AND *AEDES ALBOPICTUS* TO DIFFERENT CARBOHYDRATES IN AN OLFACTOMETER

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ABSTRACT

Eleven different carbohydrates were evaluated to determine the behavioral response of adult *Anopheles quadrimaculatus* Say and *Aedes albopictus* Skuse using an olfactometer. The carbohydrates used in the study are arabinose, fructose, glucose, maltose, melezitose, meliniose, raffinose, rhamnose, sucrose, trehalose, and turanose. The results showed that both species of mosquitoes regardless of the sex had significantly higher attraction to arabinose, maltose, meliniose, and trehalose than other 7 carbohydrates tested. Both sexes and both species responded to maltose and trehalose in considerable numbers, and the least responses were to sucrose except by male *Ae. albopictus*. These findings may provide insights to the development of more effective sugar-based toxic baits for the operational application in mosquito control programs.

Key Words: Carbohydrates, sugar feeding, *Anopheles quadrimaculatus*, *Aedes albopictus*, attractive toxic sugar bait

Anopheles quadrimaculatus Say is one of the major vectors of malaria pathogens, while *Aedes albopictus* Skuse is an important vector of dengue virus and a domestic / peridomestic pest species. Due to many reasons, such as increase of the development of resistance to insecticides, a novel control technique is urged and demanded for control of these vector mosquitoes. Attractive toxic or target sugar baits (ATSB) and bait stations are one of the new control methods. Different toxins have been used as active ingredient to make ATSB to control several species of adult mosquitoes (Xue and Barnard 2003, Muller and Schlein 2006, Muller et al. 2010). ATSB control technique is based on the sugar feeding behavior of adult mosquitoes. Sugar feeding is important for survival, reproduction, and energetics (Foster 1995). Nutrient acquisitions by adult mosquitoes are from nectar resources (Muller et al. 2011, Barredo and DeGennaro 2020). Flowers, fruits, honeydew, and seed pods of certain plants are favored and their carbohydrates could serve as potential attractants for adult mosquitoes (Muller et al. 2010a, 2011). ATSBs contain an attractive odorant and a lethal active ingredient suspended in a sugar source that mosquitoes utilize as a carbohydrate source. The attractiveness of ATSBs to compete with natural sugars available in the environments is still a big challenge. Therefore, research and development of effective attractants for ATSBs are highly demanded. Selecting the carbohydrate source based on increased mosquito response to different sugars would increase the attractiveness of ATSBs and enhance the effectiveness in operational programs. The present study was conducted to determine whether adult *An. quadrimaculatus* and *Ae. albopictus* preferentially respond to different carbohydrates and if so, could those carbohydrates be utilized to develop more effective ATBSs in the future.

Ae. albopictus and *An. quadrimaculatus* mosquitoes used in this study were received from the laboratory colonies maintained in an insectary at 27° C and 80% relative humidity in a 14:10 photoperiod (light:dark) of the US Department of Agriculture-Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. Adult mosquitoes were maintained in screened cages and provided a 10% sucrose water solution. Male and female adult mosquitoes were 5-7 days old without starvation when used in each test.

Eleven different carbohydrates, arabinose, fructose, glucose, maltose, melezitose, melibiose, rhamnose, raffinose, sucrose, trehalose, and turanose were purchased from Sigma Aldrich online. Each carbohydrate solution was prepared in methanol (99%) to have the same concentration in all. An aliquot of 100 µL of a selected carbohydrate was pipetted into a plastic vial cup (15 mm inner diameter x 9.5 mm height). Prior to use, the solution was allowed to dry for 3 minutes to remove the methanol solvent. A plastic vial cup was treated only with methanol as control.

A homemade olfactometer with 3 cages and dual-ports per cage described by Posey et al. (1998) was used to determine the response to different carbohydrates in the laboratory. Males (100) and females (100) of each species of mosquitoes were transferred to each cage (a total 400 mosquitoes/cage). The plastic cups containing each carbohydrate were loaded immediately onto an aluminum tray to hold the vials and inserted into the olfactometer ports. Six ports of the olfactometer held 6 different carbohydrates in the first run and the other 5 carbohydrates with the control were run in the next time. The test was repeated in 12 days using the Latin-square design so that each carbohydrate was tested against all the other carbohydrates and the control. Each test combination had 2 replicates. After 1 h exposure, all mosquitoes trapped in each port were separated by sex and the species and counted. The cages and ports were cleaned-up after each test and new mosquitoes from the stock cages were introduced to the olfactometer cages for subsequent test runs.

The mean percent mosquitoes entered into the ports with different carbohydrates were analyzed using multiway -ANOVA procedures (SAS 2003). Each count datum for mosquitoes trapped in different ports baited with different carbohydrates were transformed to $\log_{10}$ (n +1) before the analysis. Differences in the response of each sex of each species to different carbohydrates were compared in separate analyses using Tukey's Honestly Significant Difference (HSD) test. The level of significance in all statistical tests was $P = 0.05$.

As determined by the olfactometer bioassay, male and female mosquitoes of both species responded to all eleven carbohydrates, compared to the control. The numbers of mosquitoes responded to different carbohydrates varied by the species and the sex of mosquitoes. The most responded carbohydrates by either sex of either species were arabinose, maltose, meliniose, and trehalose (Table 1). The highest numbers of both male and female *An. quadrimaculatus* responded to maltose (16% and 18.3% respectively). *Ae. albopictus* males responded the most to trehalose (23.7%) while the females responded mostly to arabinose (35.7%). However, maltose and trehalose had high numbers of both sexes of both species although not statistically significant from the attraction by other carbohydrates in some cases (Table 1). It was surprising that the carbohydrate with the lowest response from both males and females of *An. quadrimaculatus* (1.7% and 2.7, respectively) and female *Ae. albopictus* (3.7%) was sucrose. Although not the lowest, the response of male *Ae. albopictus* (3%) to sucrose was considerably low as well.

The study findings demonstrated that 4 carbohydrates, maltose, trehalose, meliniose, and arabinose were more attractive to both *An. quadrimaculatus* and *Ae. albopictus* than the other 7 carbohydrates tested and particularly than sucrose which is the common carbohydrate used in ATSBs. Isolation of the same carbohydrates from the crops of wild-caught adult *An. quadrimaculatus* (Burkett et al. 1999) and *Ae. albopictus* (Burkett et al. 1998) indicates their feeding on the same carbohydrates in the natural environment as well. Further, a recent study demonstrated that arabinose enhanced the toxic sugar bait toxicity to *Ae. aegypti* (Linn.) adult females, but there was no impact on attractiveness of toxic sugar baits when other sugars were present (Airs et al. 2019). Supported with those evidence, the 4 carbohydrates which showed increased attractiveness in our study could be considered as an additional sugar component for the development of ATSBs, and further field evaluation is warranted.

A variety of fruit juices and chemical attractants have been incorporated into ATSBs or TSBs (toxic sugar bait) and evaluated for control of adult mosquitoes (Muller et al. 2010, Xue et al. 2008, Fiorenzano et al. 2017, 2017a). However, the attraction from natural fruits, fruit juices and their extracts did not show a strong attraction. The common chemical attractants (CO2 and Octenol) incorporated with TSBs have increased the attraction of adult female mosquitoes and improved the control efficacy (Fiorenzano et al. 2017a). Toxic sugar baits use sugar as a phagostimulant to induce ingestion of an oral toxin, but sugar alone is not an effective attractant (Fiorenzano et al. 2017). Most ATSB products use brown sucrose which does not have significant attraction. The development and application of more attractive and effective toxic sugar baits and bait stations would provide another useful tool to mosquito management programs and public health officials to continue to combat mosquitoes and mosquito-borne diseases.

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Table 1. Mean percent of adult *Anopheles quadrimaculatus* and *Aedes albopictus* attracted to each type of carbohydrates in 1 hour evaluated by olfactometer bioassay

	Anopheles quadrimaculatus		*Aedes albopictus*	
Carbohydrates	Male	Female	Male	Female
arabinose	12.7 ab	8.0 bc	10.7ac	35.7a
fructose	3.3 b	6.7 bc	2.7 c	7.3 c
glucose	2.3b	7.3bc	4.3 c	7.0 c
maltose	16.0a	18.3ab	12.3abc	31.0ab
melezitose	7.7ab	5.7bc	8.3bc	7.0c
meliniose	12.7ab	8.0bc	19.0ab	22.0abc
raffinose	2.7b	7.7bc	4.3c	9.3c
rhamnose	4.0b	7.0bc	3.0c	3.7c
sucrose	1.7b	2.7c	3.0c	3.7c
trehalose	11.7ab	13.0abc	23.0a	14.0c
turanose	2.7b	6.3c	6.7c	7.3c
Control	0.3ab	0.7ab	0.7ab	0.3ab

Mean percent in each column and row followed by the same letter are not significantly different (Tukey's HSD, $P > 0.05$).

EFFICACY OF AN ESSENTIAL-OIL ADULTICIDE FORMULATION, BIGSHOT MAXIM CONCENTRATE, AGAINST RESISTANT AND SUSCEPTIBLE STRAINS OF *AEDES AEGYPTI* IN A WIND TUNNEL

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ABSTRACT

An essential-oil adulticide formulation. BigShot Maxim Concentrate (14% cedarwood oil, 0.53% thyme oil, and 0.25% cinnamon oil), was evaluated using a wind tunnel against pyrethroid resistant (Puerto Rico, PR) and pyrethroid susceptible (Orlando, ORL) colony-reared strains of *Aedes aegypti* to determine whether the product could be used in operational mosquito control to supplement dwindling efficacy of pyrethroid formulations. The product was sprayed at 0.5x (146 mL/ha), 1.0x (291 mL/ha), and 2.5x (731 mL/ha) the maximum application rate through a ULV nozzle. After application, mortality was checked at 1 and 24 h. The 24 h mortality for the ORL strain was 85.9% ±5.0, 98.7% ±1.3, and 99.2% ±0.8 at the three application rates, respectively. In contrast, mortality at 24 h post exposure for the PR resistant strain was significantly lower, 26.4% ±6.5, 35.2% ±8.0, and 45.1% ±8.0, at the three application rates, respectively. Results suggest that the essential-oil formulation could be moderately effective against a resistant strain of *Ae. aegypti* if applied at very high rates and would likely need to be reapplied frequently, and target populations monitored for evolution of resistance to cedarwood and other essential oils.

Key Words: *Aedes aegypti*, essential oils, resistance, wind tunnel, mosquito control

Aedes aegypti L. (Diptera: Culicidae) is an aggressive daytime biter and major vector of yellow fever, Zika, and dengue viruses to humans (Braack et al. 2018). Due to increasing resistance to widely used pyrethroids, control of this species with standard adulticide formulations is becoming more difficult (Hemingway & Ranson 2000). One strategy to mitigate such resistance is to pivot to alternative adulticides formulated with natural plant-based products which are becoming increasingly available for public health mosquito control (Sukumara et al. 1991, Prabhakar & Jebanesan 2004). In this study we investigated the comparative efficacy of a plant-based essential-oil adulticide formulation, BigShot Maxim Concentrate (PreVasive USA, Oakwood, GA), against both susceptible and resistant strains of colony-reared *Ae. aegypti* in a wind tunnel at three application rates to determine whether this formulation could be considered for use in the field against resistant populations of this species. BigShot Maxim Concentrate contains three plant-based active ingredients, cedarwood oil (14%), thyme oil (0.53%), and cinnamon oil (0.25%), and is labeled for use in a variety of application techniques including ULV, barrier treatments, and misting systems.

Eggs from two strains of *Ae. aegypti*, the susceptible Orlando (ORL) strain and the resistant Puerto Rico (PR) strain, were obtained from the US Department of Agriculture (USDA), Agricultural Research Service Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) in Gainesville, FL and reared in colony at Anastasia Mosquito Control District (AMCD) insectaries maintained at 26.7 ± 1 °C, 80 ±10% relative humidity (RH), and 14L:10D photoperiod. Adult mosquitoes were provided a 10% sucrose solution *ad libitum* and blood-fed using a restricted chicken to procure eggs. Larvae were reared in 56 cm x 43 cm x 8 cm plastic trays on a diet of powdered tropical flakes fish food (purchased from St. Augustine feed store) administered in a 1:6 food:water slurry.

To investigate the efficacy of the essential-oil adulticide formulation in a wind tunnel we developed a simple disposable bioassay sentinel cage as shown in Fig. 1 that was sized to allow 5 cages at a time to be placed in the wind tunnel test section. We assayed three application rates of the adulticide in the wind tunnel, with three trials using the susceptible ORL strain and the resistant PR strain of *Ae. aegypti* for each of the three application rates: 146 mL/ha (minimum label rate), 291 mL/ha (maximum label rate), and 731 mL/ha (approximately 2.5 times the maximum label rate). These rates corresponded to cedarwood oil application rates of 20.4, 40.7, and

102.3 mL/ha, respectively. Spray volume to be dispersed through wind tunnel (*SV*, µL) was determined considering the cross sectional area of the wind tunnel (*Wa*, m^2) using the following equation:

$$SV = \frac{AR \times SW \times W_a}{10H}$$

Where:
AR = Application rate, mL/ha
SW = Swath width, m
H = Height above ground up to which spray is considered as dispersed during ULV applications, m.

For this study, *SW* was 91 m, *Wa* was 0.28 m^2, and *H* was 3.0 m. For each trial for each strain for each application rate we assayed 5 bioassay cages simultaneously, with each bioassay cage containing 25-30 five-to-ten-day old non-blood fed adult female mosquitoes of one strain.

We used a wind tunnel based on that described by Bibbs et al. (2020) with an exhaust pipe (168 cm long, 14.7 cm diam.) equipped with a suction fan at the end of the test section of the wind tunnel to draw through and remove the sprayed pesticide. The essential-oil formulation was sprayed from the opposite end of the exhaust section using the Air-Shear ULV nozzle (Model: Terminator, ADAPCO, Sanford, FL) supplied with 100 psi of air to propel the product. After each trial, we allowed a 1 min period to lapse to allow residual product to clear the wind tunnel before the 5 bioassay cages were removed and the next 5 cages set up for the next trial. Before the set of trials for each application rate, the ULV nozzle was flushed with 70% isopropanol alcohol and then with water. After this flush routine, 5 bioassay cages of each species, in turn, were introduced and sprayed with water through the ULV nozzle to verify that the system was clear of adulticide and to simultaneously establish that the setup itself did not induce mortality in the bioassay system.

After each trial all five bioassay cages were removed from the wind tunnel and provided a cotton ball saturated with a 10% sucrose solution, and stored in separate control and treatment incubators maintained at 26.6 °C and a 14L:10D photoperiod where mortality was recorded at 1 and 24 h post-application. JMP version 14 (SAS Institute Inc. Cary, NC) was used to conduct statistical analysis of the mortality data. Mortality data were determined to be non-normal. We conducted a one-way non-parametric analysis of variance Wilcoxon test at the 95 % significance level, and compared means using the Wilcoxon Each Pair comparison tool of the Nonparametric Multiple Comparison procedure tested at the 95 % significance level.

Results are displayed in Table 1 which shows the mean mortality and standard errors for each strain at each application rate at 1 and 24 h. Mortality was significantly higher in the susceptible ORL strain at both 1 h (X^2 = 28.87, df = 1, $P < 0.0001$) and 24 h (X^2 = 25.89, df = 1, $P < 0.0001$) post-application time periods.

This study demonstrates that an essential-oil based adulticide containing cedarwood, thyme, and cinnamon extracts (BigShot Maxim Concentrate) was significantly less effective against the resistant PR strain of *Ae. aegypti* compared to the susceptible ORL strain. Importantly, the maximum label application rate of 291 mL/ha (40.7 mL cedarwood oil/ha) or higher was needed to induce more than 86% mortality in the susceptible ORL strain, and neither the maximum nor the 2.5 times maximum application rate resulted in greater than 45% mortality for the resistant PR *Ae. aegypti* strain.

Our results contrast with those published in the literature for cedarwood oil, the main active ingredient of the BigShot Maxim Concentrate, which has been shown to cause high mortality in arthropod pests (Eller et al. 2014, Khanna & Chakreaborty 2018). In another study, a botanical insecticide containing 25.3% cedarwood and 12.7% cinnamon oil (NatureCide Pest Management) was shown to cause 100% mortality against colony reared

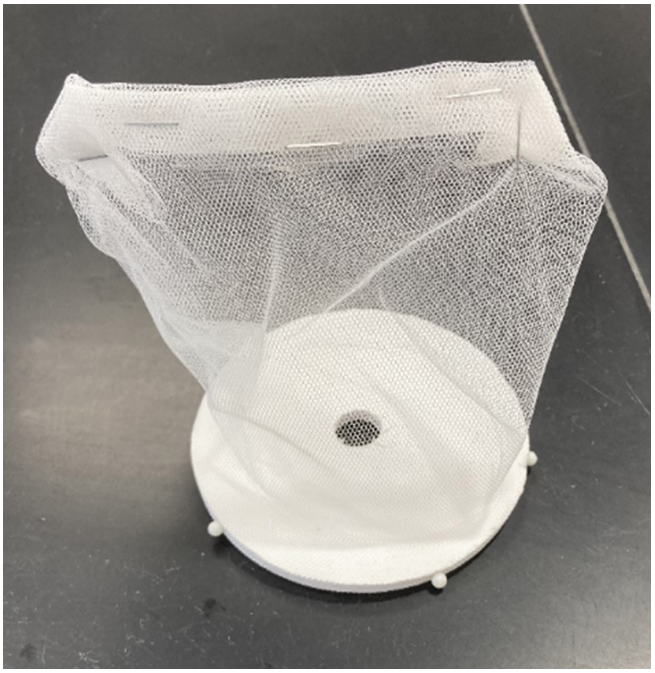

Figure 1. Sentinel mosquito cage constructed of a 10 cm diam. foam board with a 1 cm hole in the middle fitted with 30 cm wide x 15 cm high nylon tulle netting secured with 6 pins, with the top and side seams folded and stapled. For each bioassay, 15-20 5-10 d old non-blood-fed females were introduced through the hole and secured with a cotton ball.

Table 1. Mean percent 1 h and 24 h mortality and standard error (SE) at three application rates of BigShot Maximum Concentrate against 5-10 d old non-blood-fed females from the susceptible Orlando (ORL) and resistant Puerto Rico (PR) strains of *Aedes aegypti* in a wind tunnel.

	1 h mortality (mean % ±SE)		24 h mortality (mean % ±SE)	
Application Rate (mL/ha)	ORL strain	PR strain	ORL strain	PR strain
146	64.5 ±6.7*	3.5 ±1.5	85.9 ±5.0*	26.4 ±6.5
291	78.4 ±5.8*	14.4 ±3.8	98.7 ±1.3*	35.2 ±8.0
731	86.9 ±6.7*	31.7 ±9.1	99.2 ±0.8*	45.1 ±8.0
Control (water only)	0	0	0	0

*Significant difference ($P < 0.0001$) between strains for the same time period on this row.

ORL *Ae. aegypti* in a ULV treatment at a concentration of 70 ml/L [2.1 mL cedarwood oil/ha] (Bibbs et al. 2019). Bangonan et al. (2021), found in laboratory bioassays that BigShot Maxim Concentrate was effective at killing colony reared susceptible *Ae. aegypti*, *Culex quinquefasciatus* Say, and *Anopheles quadrimaculatus* Say, compared to a permethrin control – although, similar to the current study, at an application rate higher than the maximum label application rate of 40.7 mL cedarwood oil/ha.

The formulation of cedarwood, thyme, and cinnamon oils in the current study is a natural insecticide exempt from the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) 25B, and could possibly be applied more frequently than non-25B pesticides to offset the significantly lower efficacy against resistant strains of *Ae. aegypti*. However, further evaluation is needed to determine whether more frequent applications (a) are cost effective, (b) will actually reduce natural populations of resistant *Ae. aegypti*, and (c) will not cause undue non-target or other negative collateral effects.

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EVALUATION OF POTENTIAL SPATIAL REPELLENCY OF CONTACT REPELLENTS AGAINST *AEDES AEGYPTI* (L.) IN A WIND TUNNEL

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ABSTRACT

The use of arthropod repellents is an important personal protective measure against vector-borne diseases. For contact repellents, the recommendation to apply repellents to all exposed skin could be relaxed if the repellent exhibits spatial repellency. In 2019, we evaluated four contact repellents containing a mixture of geraniol and soybean natural oils, N,N-diethyl- 3-methyl-benzamide (DEET), 2-(2hydroxyethyl)-1-piperidine carboxylic acid 1-methylpropyl ester (Picaridin), and p-Menthane-3,8-diol (PMD) for their potential as spatial repellents against cohorts of irradiated and non-irradiated laboratory reared *Aedes aegypti* (L.) and irradiated *Ae. aegypti* females exposed to a radiation dose sufficient to sterilize (50 Gy). Evaluations were conducted in a modular wind tunnel, which provided mosquitoes the option to move within 15 minutes to the repellent side containing a repellent or to an attractant side containing BG lure. Mosquitoes on each side were counted and percent calculated based on the number of mosquitoes released for each test. The repellent containing PMD had significantly more non-irradiated mosquitoes on the attractant side than on the repellent side, indicating that it repelled non-irradiated mosquitoes. Picaridin had significantly more irradiated mosquitoes on the attractant side than on the repellent side indicating that it repelled irradiated mosquitoes. A minor change in behavior of irradiated and non-irradiated mosquitoes by these repellents can only create a false sense of protection. These results emphasize to strongly follow available guidance that the contact repellents tested in this study should be applied to all exposed skin.

Key Words: Repellency, irradiated mosquitoes, SIT, topical repellents, personal protection

Common vector-borne disease prevention measures are aimed at reducing vector populations, although some are aimed at avoiding vector-human contact. Norris and Coats (2017) described all mosquito population control measures aimed at either prevention of host-seeking or prevention of biting by the vectors. The mosquito-biting rate represents a second-order parameter in overall vectorial capacity of a mosquito species, therefore, it is theoretically possible to drastically lower the spread of mosquito-borne diseases by disrupting mosquito host-seeking and biting (Norris and Coats 2017). Protection against vector-borne diseases needs both community and personal, i.e., individual efforts. Although most mosquito problems cannot be controlled by individual efforts, however, in a localized area, an individual can have a large impact through the use of personal protective measures. Use of arthropod repellents, contact or spatial, is one of the important individual efforts for the protection against disease vectors. Achee et al. (2012) have collectively termed these techniques as mosquito behavior modification methods of vector-borne disease control.

Contact or topical repellents have been in use for a long time. These repellents, as defined by Farooq et al. (2021) are either applied to skin or to clothes to prevent mosquitoes or other arthropods from landing and staying on the treated surface and biting an individual. According to Norris and Coats (2017), mosquitoes must come into close proximity or direct contact with the surface treated with contact or topical repellents, in order to be repelled. The use of these topical repellents is often compromised by reluctance of individuals due to an unpleasant smell, oily residue, and/or dermal irritation (Lloyd et al. 2013). On the other hand, spatial repellents go a step forward to prevent the vector from coming in contact with the host by creating a barrier between the vector and the host (Dame et al. 2014; Farooq et al. 2021).

When using contact repellents, it is recommended to evenly apply the product to all exposed skin, which realistically may not be practical. Repellent labels also advise not to apply the product close to the eyes and mouth. A contact repellent having some spatial repellency may help protect exposed areas of skin adjacent to treated skin, which are left untreated due to label recommendations or for any other reason. Currently, separate formulations are available as contact and spatial repellents. As emphasized by Achee et al. (2012), one way to overcome the limited

availability of new repellent compounds is to find new means of utilizing existing compounds.

The sterile insect technique (SIT), an area-wide control method that is based on the release of male insects sterilized by ionizing radiation into the target area to reduce the reproduction of a natural population of the same species, has been widely used for many decades to control plant pest species (Knipling 1955). The SIT is rapidly evolving as an additional tool for mosquito control, offering an efficient and more environmentally friendly alternative to the use of insecticides (Bellini et al. 2013; Gouagna et al. 2020). This technique is currently being evaluated at a large scale before full-scale adoption (Sypes 2021). Because SIT is insecticide-free and species-specific, it is considered an environmentally friendly or neutral method, which has led to its increased implementation worldwide (Enkerlin 2005) following one of four strategies such as eradication, suppression, containment, or prevention (Hendrichs et al. 2005). Cunningham et al. (2020) has shown that blood feeding by female *Aedes aegypti* was reduced due to irradiation. Xue and Linthicum (2020) reported that the host avidity (host attacking/min) in irradiated female *Ae. aegypti* (L) mosquitoes was significantly lower than the attacking rate in the non-irradiated mosquitoes, and DEET (15%) on volunteer forearm provided 1.5-2.0 hrs longer protection time than against the non-irradiated female mosquitoes. However, we still do not know whether the contact repellents provide potential spatial repellency against irradiated and non-irradiated female *Ae. aegypti* mosquitoes.

We evaluated four commercially available contact repellents for their potential spatial repellency against irradiated and non-irradiated laboratory reared 5-7 days old female *Ae. aegypti* in a wind tunnel. Non-irradiated mosquitoes were reared at Anastasia Mosquito Control District (AMCD), St. Augustine, Florida (FL) insectary with conditions maintained at 26.6 ± 1°C temperature, 70.0 ± 10% relative humidity and 14:10 (L:D) photoperiod. Mosquitoes irradiated with 50 Gy as described by Moreno et al. (2021) were obtained from Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, FL. These mosquitoes were received by AMCD staff and maintained similarly as the non-irradiated ones.

The four commercial repellent products tested were: Cutter Natural (5% geraniol and 2% soybean oil), Cutter Skinsations (7% DEET), SC Johnson Off (5% Picaridin), and SC Johnson Botanical (10% p-Menthane-3,8-diol [PMD]). Based on the evaluation scheme by Grieco et. al. (2005), a modular wind tunnel at AMCD (Figure 1), described in detail by Bibbs et al. (2020) was configured to work as an olfactometer. The wind tunnel comprised of a 52 cm wide x 52 cm tall x 156 cm long test section and a 47 cm long bifurcation module on the upwind side of the test section with a screen separating them. The screen between the two sections was made to hold two 32 cm long and 14 cm diameter glass tubes. The downside side of this tube has a funnel, while upwind side has a screen so when the mosquitoes enter through the funnel, they are trapped in the tube. The wind tunnel has two odor release chambers on top of the wind tunnel (Figure 1). A

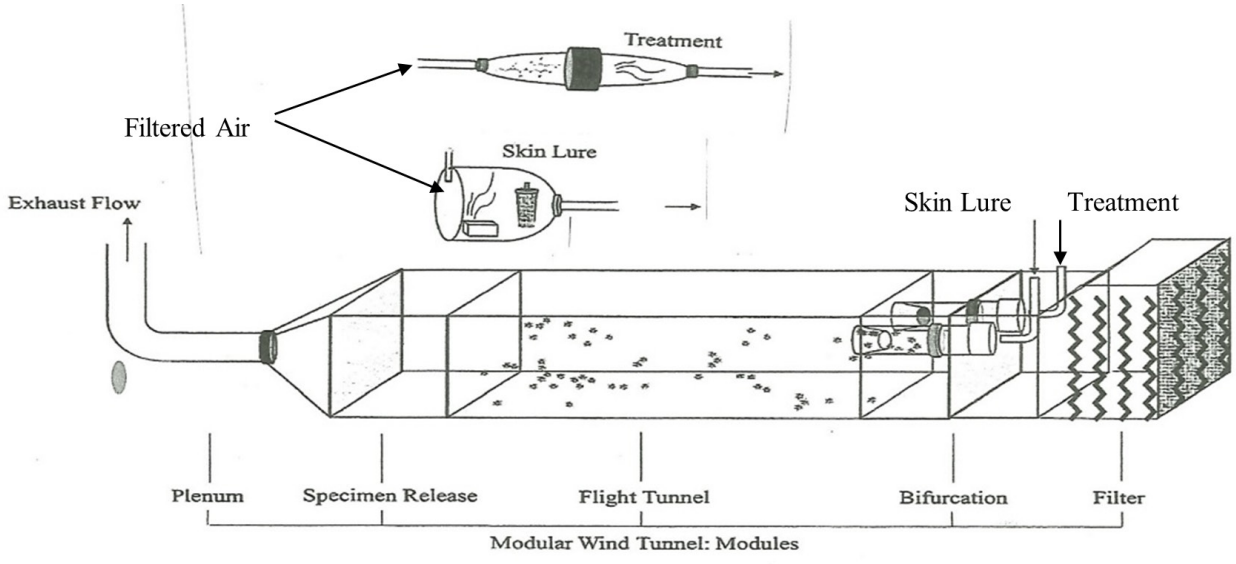

Figure 1. Layout of the wind tunnel.

controlled volume of clean and dry air flows through these chambers into upwind side middle of one each of the glass tubes. Air moving through these chambers carry fumes to one of the glass tubes. The wind tunnel also contains a filter module on upwind side of bifurcation chamber to trap any dust or particulate in the air entering the tunnel. On the downwind of the test section is an insect release chamber which connects to an exhaust pipe. A blower at the end of the exhaust pipe causes air movement through the tunnel at a maximum speed of 1.5 km/h and removes air from test section to outside the building.

For the tests, 1 ml of arthropod repellent was poured onto small pieces of filter paper at the bottom of 59.1 mL portion container. The BG lure cartridge (Biogents USA, Moorefield, WV) was placed in one of the odor release chambers as an attractant, i.e., attractant side and one of the repellents was placed in the other chamber, i.e., repellent side. The sides containing an attractant and repellent were maintained throughout the study to protect the attractant side from contamination by the repellents. Five tests, each replicated three times were conducted. One of the five was a control test with BG lure on the attractant side and nothing on the repellent side. Four tests were conducted with BG lure on attractant side and filter paper soaked with repellent on the repellent side. The olfactometer was cleaned and decontaminated between repellents. For each trial run, one hundred 5-7 days old, 24-h starved female *Ae. aegypti* were released inside the chamber on the downwind side of the test section. Fifteen minutes after release, mosquitoes in each tube were counted. The difference between mosquitoes in two tubes indicated the repellency of the product and the number of mosquitoes on each side was converted to the percent of mosquitoes in that side based on the total number of mosquitoes released for each test.

Analysis of variance (ANOVA) was performed to assess the significance of difference in percent of mosquitoes in each section, between repellents and control, and between repellents using JMP edition 14 (SAS Institute Inc., Cary, NC). The means were compared using Tukey's multiple comparison test at 95% level of significance.

Analyzing all the data by averaging percentage of mosquitoes on two sides and for the two types of mosquitoes used, there was not a significant effect of repellents on mosquito behavior ($p = 0.579$, $df = 4$). When all the data were combined for repellents, control and two types of mosquitoes, the difference in mosquitoes moving to the repellent side or to the attractant side was significant ($p = 0.026$, $df = 1$). For non-irradiated mosquitoes, there was no significant difference in the percent of mosquitos moving to the attractant side ($p = 0.95$) or to the repellent side ($p = 0.94$), between control and repellents and within repellents. Similarly, there was no significant difference in percent of irradiated mosquitoes moving to the repellent side ($p = 0.39$), between control and repellents, and within repellents. However, significantly more irradiated mosquitoes moved to the attractant side from SC Johnson Off containing Picaridin and Cutter Natural containing natural oils than control and other two repellents. As shown in Figure 2, SC Johnson Botanical containing PMD had significantly more non-irradiated mosquitoes on the attractant side than on the repellent side indicating that it

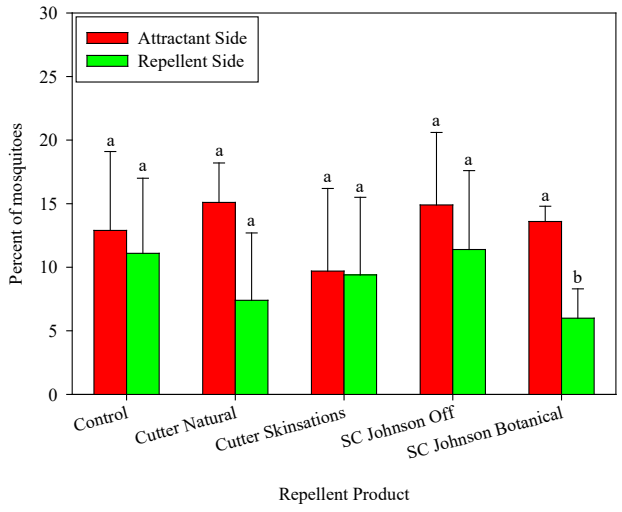

Figure 2: Percent of non-irradiated mosquitoes moving to the repellent and attractant sides. The different letters on bars indicate a significant difference between repellent and attractant sides at $\alpha = 0.05$.

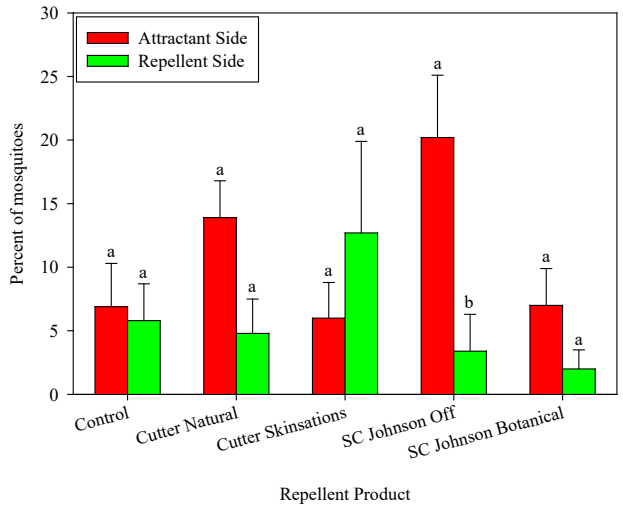

Figure 3: Percent of irradiated mosquitoes moving to the repellent and attractant sides. The different letters on bars indicate a significant difference between repellent and attractant sides at $\alpha = 0.05$.

repelled non-irradiated mosquitoes. SC Johnson Off had significantly more irradiated mosquitoes on the attractant side than on the repellent side indicating that it repelled irradiated mosquitoes (Figure 3). Although the difference in percent of mosquitoes between repellent and attractant side from Cutter Skinsations containing DEET was not significant, irradiated mosquitoes were attracted to the repellent side (Figure 3).

The data indicated that irradiated mosquitoes showed significant spatial repellency to SC Johnson Off and some level of repellency to Cutter Natural and SC Johnson Botanical, but an attraction to Cutter Skinsations. Non-irradiated mosquitoes showed some spatial repellency to SC Johnson Botanical and Cutter Natural but not to other repellents tested. This lack of response or limited response to the repellents tested against these mosquitoes indicates need for more cautious approach towards using these arthropod repellents as contact repellents.

In conclusion, there was a small change in behavior of irradiated mosquitoes and non-irradiated mosquitoes when exposed to three of the four repellents tested. However, this change in behavior does not seem to be strong enough to keep the mosquitoes away from the human individual. Instead, these results could reveal a false sense of protection. These results emphasize to strongly follow available guidance when using the repellents tested in this study as contact repellents, because they should be applied to all exposed skin areas of the body. The repellents on one part of the body will not deter mosquitoes contacting other parts that have not been treated. Little impact on behavior of irradiated mosquitoes should not let us relax on the use of contact repellents due to their small proportion in the overall mosquito population.

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RESIDUAL EFFECTS OF BIFENTHRIN SPRAYED ON PLANT FOLIAGES AGAINST *AEDES ALBOPICTUS* AND *APIS MELLIFERA* IN NORTH CENTRAL FLORIDA

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Guest Editor: Aaron M. Lloyd

ABSTRACT

A field study was conducted to test bifenthrin as a barrier treatment for its residual effects on adult mosquitoes *Aedes albopictus* and honey bees *Apis mellifera* in Gainesville, Florida. Plant foliage was treated with an American LongRay misting sprayer machine at the label rate of 0.318 mL/m². Treated plant leaves were then collected at 24 hr, one wk, and two wk post-treatment for laboratory bioassays against adult *Ae. albopictus* and *A. mellifera*. The mortalities of the mosquitoes and honeybees were significantly higher after exposures to the bifenthrin-treated plant leaves at 24 hr post-treatment, than the mortalities at one wk and two wk post-treatments. There were no significant differences in the mortalities of mosquitoes and honeybees exposed to treated plant leaves at one wk and two wk post-treatment. Also, the results showed that the treated plant leaves away from the spray path resulted in significantly high mortalities of both species at 24 hr post-treatment than the mortalities at 8 m and 11 m at one wk and two wk post-treatment. The commercial product of Talstar P (bifenthrin) sprayed on plant foliage resulted in significantly higher mortalities of mosquitoes and honeybees at 24 hr post-treatment at the 5 m distance. There was no significant residual efficacy of the product one week after post-treatment at any distance.

Key Words: *Aedes albopictus*, *Apis mellifera*, bifenthrin, vegetation, barrier treatment

Seven commercial barrier treatment products and compounds against adult mosquitoes have been evaluated in the laboratory (Qualls & Xue 2013) and field (Stoops et al. 2019, Bibbs et al. 2020), of which bifenthrin is the most effective insecticide for barrier sprays. Bifenthrin applied to vegetation and has provided effective control of adult mosquitoes in the semi-field and residential areas (Bibbs et al. 2016, Cilek & Hallmon 2008, Qualls et al. 2012, Lloyd et al. 2021). This method has been adopted as a part of operation control by the Anastasia Mosquito Control District (AMCD) in St. Johns County, Florida (Qualls et al. 2013). The residual effects of bifenthrin on plant leaves against adult mosquitoes could last for about 3-4 weeks in the semi-field and field (Doyle et al. 2009, Fulcher et al. 2015). The efficacy of bifenthrin on vegetation is impacted by environmental conditions (Allan et al. 2009). Laboratory bioassays showed that bifenthrin caused significant mortality for honeybees after direct contact (Qualls et al. 2010, Sanchez-Arroyo et al. 2019, 2021). So far, we do not know whether the barrier spraying of bifenthrin on vegetation in the residential areas has any residual impact on the honeybee, *Apis mellifera* Linnaeus when we aim to control the container-inhabiting mosquitoes, *Aedes albopictus* Skuse. The present report is about the residual impacts of bifenthrin barrier treatment on vegetation against *Ae. albopictus* and *A. mellifera* around a residential subdivision in north central Florida.

The eggs of *Ae. albopictus* mosquitoes were obtained from the Anastasia Mosquito Control District and reared and kept in the insectary of Urban Entomology Laboratory, University of Florida (UF), Department of Entomology and Nematology (DEN), Gainesville, Florida. Adult female mosquitoes at 3-5 days old were used in the bioassays. The adult honey bees, *A. mellifera* at 5-7 days old were provided by the Bee Laboratory at the UF/DEN.

Whitney Mobile Home Park, a residential subdivision (29°43'56.89"N, 82°22'29.29"W) located in the northern part of Gainesville, Florida was selected for the field experiment. This area was chosen because of its large interior park-like setting, surrounding dense vegetation with similar habitats, and suitable environment for barrier treatments. There are a few pine trees and dense ground vegetation (major species were *Melampodium paludosum* Melanie and *Duranta erecta* L. (Golden dewdrop)) around the subdivision.

A misting spray machine (model 3WC-30-4P, American LongRay, San Francisco, CA) was used for the study. The sprayer is powered by a 6.30Kw 16-liter diesel engine with 4 adjustable spray nozzles. The flow rate can be continuously varied from 0.83 to 5 L/min. The engine can be turned on and off using a remote control up to 5.5 meters away from the sprayer. The machine was calibrated prior to the treatment. The flow rate was set at 4.7 L/min with a median droplet size diameter (Dv0.5) of 107.5 µm and a mean droplet velocity of 6.9 m/sec. The unit along with the external tank was mounted into a Polaris Ranger (4x4). The sprayer nozzle heads were set at 360° vertically and 330° laterally in order to create a spray pattern 3 m high and 3.1 m wide, respectively, when the Polaris Ranger was stationary. A 168.8 L external tank was added to the unit to ensure the spraying mission would be complete without refilling the tank.

A commercial product Talstar P (A.I. 7.9% of bifenthrin, FMC Corporation, Philadelphia, PA) was diluted to 7.8 mL of formulation per liter of well water and applied at the label rate of 0.318 mL/m². During the application, the Polaris Ranger carrying the spray machine with the nozzle was at approximately 0.5 m from the nearest vegetation and the driving speed was 8 Km/h. The application rate resulted in 0.025 g A.I./m² of the vegetation surface at the 5 m distance away from the machine. The control plot was untreated and about 500 m away from the treated site. Runoff of insecticides was not observed during the treatment.

Residual effects of bifenthrin treatment on the vegetation were measured using the leaf bioassay method (Xue 2008). Leaf clippings from bushes of treated and untreated sites were used for the bioassays against *Ae. albopictus* and *A. mellifera*. Nine leaves (six *M. paludosum* and three *D. erecta*) were excised from three different rows (3 leaves from each row) 3 m apart from each other at 5 m, 8 m, and 11 m from the line of travel of the swath of the sprayer. Each sampled leaf had a similar surface area, thickness, and waxiness. Upon return to the laboratory, each leaf was contained in a 60 mL cup. For each bioassay, ten adult female *Ae. albopictus* mosquitoes (3–5 days old) and ten adult *A. mellifera* honeybees at 5-7 days old were knocked down using carbon dioxide (CO_2) anesthetization and placed in the respective leaf-cup through forceps manipulation. Cups with cotton balls, saturated with 10% sucrose solution for mosquitoes and 50% sucrose for honeybees, were stored in a temperature-controlled room (24°C). Mortality as indicated by complete non-response to the stimulus, was recorded at 24 hr of continuous exposure. Each trial was composed of 3 cups for the treatment and 3 cups for the control. Similar bioassays were carried out weekly after the treatment. When the mortality in the treatment group was less than 50% the weekly experiment was stopped. The trial was repeated three separate times.

The treatment mortalities were corrected for any control mortalities using the Abbott formula (Abbott 1925) and the data were analyzed by computer software. One-way and Two-way ANOVA were used appropriately to compare the mortalities among the two species for 3 different post-treatment time periods, and three different distances.

The experiment was stopped at three wk post-treatment due to the detection of low mortality (less than 50%) for both species of insects in the treatment group. The data analysis (Table 1) showed that the treatment resulted in significant differences in the mortalities of mosquitoes, *Ae. albopictus* ($F = 5.520$, $df = 10$, $P < 0.05$) and the honeybees *A. mellifera* ($F = 6.613$, $df = 10$, $P < 0.05$) after exposed to the treated plant leaves. The mortality at 24 hr was 45.6 ± 20.89 for mosquitoes and 60.3 ± 12.65 for honey bees and the differences were significant compared to corresponding controls. The mortalities of both species at 24 hr were significantly higher than the mortalities in

Table 1. Mean mortalities (% ±SD) of *Aedes albopictus* and Apis *mellifera* exposed to foliage sprayed by bifenthrin at 24 hr, one wk, and two wk post-treatment

Post-treatment period	*Aedes albopictus*		*Apis mellifera*	
	Control	Treated	Control	Treated
24 hr	0.7±0.06	45.6 ±20.9a	0.0	60.3 ±12.7b
one wk	0.0	17.8 ±31.1c	0.0	18.9 ±35.2c
two wk	0.3±0.03	13.3 ±16.6c	3.3±0.3	10.0 ±15.0c

Different letters in the column indicate significant differences.

the one wk and two wk post-treatment, but the mortalities did not show any significant differences between the one wk and two wk post-treatment.

One way ANOVA showed that there were significant differences in the mortalities of mosquitoes and honeybees in the 5 m distance at 24 hr post-treatment, compared to the mortalities in the 8 m and 11 m (Table 2), but the mortalities at the one wk and two wk post-treatment were not significantly different between the 8 m and 11 m distances. Two-way ANOVA showed that there was no statistically significant interaction between the effect of species (honeybees and mosquitoes) and the distances on mortalities ($F_{2,12} = 0.289$, $P = 0.754$).

Plants are a major part of the mosquito ecosystem (Xue 2008a) and the application of insecticides to perimeter vegetation for the purpose of controlling adult mosquitoes in backyards and other recreational areas has been confirmed to be effective control methods (Stoops et al. 2019; Richards et al. 2017). Bifenthrin has been approved to suppress mosquito populations in treatment areas and previous leaf bioassays have revealed that bifenthrin-treated leaves exhibited > 70% knockdown/mortality against laboratory-reared female *Ae. albopictus* and *Culex quinquefasciatus* for 4 weeks (Cilek 2008). Bifenthrin (0.08%) and lambda-cyhalothrin (0.1%) as barrier treatments at their maximum label concentrations has significantly reduced *Aedes* spp. population up to six wks post-treatment (Cilek 2008, Trout et al. 2007).

Trapping results (CDC light trap) in a study using bifenthrin as a barrier treatment against *Ae. aegypti*, conducted in St. Augustine, FL, showed a 77% mean reduction in adult mosquito population up to four wks post-treatment and the laboratory leaf bioassays revealed an average mortality of 80% at 2.7 m and 51% at 5.5 m for five wks post-treatment (Fulcher et al 2015). Leaves collected from the treated areas caused significantly higher mortality at distances closer to the sprayer, though the distance and coverage of bifenthrin application was effective up to 5 m (Fulcher et al. 2015). Our study showed similar results for mosquitoes and honeybees exposed to the bifenthrin treated leaves.

Table 2. Mean mortalities (% ±SD) of *Aedes albopictus* and *Apis mellifera* exposed to foliage sprayed with bifenthrin at different distances and different post-treatment time periods

Distance	*Aedes albopictus*		*Apis mellifera*	
Meter	Control	Treated	Control	Treated
24 hr post-treatment				
5	0.7±0.03	41.0±25.7a	0	48.7±36.8a
8	0	22.3±12.9b	0	25.7±33.5b
11	0	13.3±13.5b	0	26.7±32.8b
One wk post-treatment				
5	0	66.7±20.3a	0	64.3±25.0a
8	0	32.3±31.1b	0	49.0±24.3b
11	0	27.7±27.3b	0	29.0±29.8b
Two wk post-treatment				
5	0	66.7±28.9a	0	59.0±23.1a
8	0	37.0±36.1b	0	43.3±33.9b
11	0.3±0.03	29.0±33.0b	3.3±0.3	26.7±37.9b

Different letters in the column (within the sampling time) indicate significant differences.

The efficacy of bifenthrin as a barrier treatment on vegetation varied with the species of plants and insect species, application rates of the insecticide, and many other environmental conditions (Allan et al 2009, Britch et al. 2009). The impact of bifenthrin on honeybees, *A. mellifera*, was shown to be affected by both dose and exposure time (Qualls et al, 2010; Sanchez-Arroyo et al. 2019, 2021). Application dose of 35 µg/mL resulted in 100% bee mortality in the laboratory bioassays (Sanchez-Arroyo et al. 2019). In a study in which bifenthrin was applied at different concentrations to common landscape vegetation of *M. paludosum* and *D. erecta* L. (Golden dewdrop), honey bee mortality was significantly higher at high application rates, compared to the mortality at low application rates after 24 hr exposure to the treated vegetation (Qualls et al. 2010). It was not a surprise that the bifenthrin-barrier treatment on plant leaves against *Ae. albopictus* mosquitoes resulted in a high mortality of non-target honey bees during this experiment.

In summary, bifenthrin as a barrier treatment on vegetation provide effective control of the container-inhabiting *Aedes* mosquitoes, but also showed a high mortality impact on non-target honeybees at a short distance and a short time direct exposure. Fortunately, the foraging activity of honeybees in the natural environment is not around residential areas, unlike that of container-inhabiting *Aedes* mosquitoes. This may provide less opportunity for honeybees to be exposed to bifenthrin-treated vegetation when we plan to control the container-inhabiting *Aedes* mosquitoes to protect the residents from mosquito pressure. Also, this indicates that we have to take caution and limit the impact on nontarget honeybees when we select the barrier-spraying method to control target mosquitoes at residential areas.

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