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Susceptibility of the southern house mosquito, *Culex quinquefasciatus*, in East Baton Rouge Parish to larval insecticides

Nicholas Alexander DeLisi

Louisiana State University and Agricultural and Mechanical College

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SUSCEPTIBILITY OF THE SOUTHERN HOUSE MOSQUITO, *CULEX*
QUINQUEFASCIATUS, IN EAST BATON ROUGE PARISH TO LARVAL
INSECTICIDES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Entomology

by
Nicholas Alexander DeLisi
B.S., University of Utah, 2013
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ABSTRACT

Mosquito control districts in Louisiana focus their efforts on *Culex quinquefasciatus*, the primary vector of West Nile virus in the southern United States, with rigorous larvicide treatments. However, the development of resistant populations of *Cx. quinquefasciatus* in response to extensive insecticide application has been demonstrated repeatedly. Examining changes in insecticide susceptibility and larvicide efficacy in real world scenarios can help inform mosquito control districts as to whether or not their treatments are killing mosquitoes. We hypothesized that frequent larvicide applications for the control of mosquitoes in East Baton Rouge Parish had lowered susceptibility of wild *Cx. quinquefasciatus* to insecticides, and that treatment in real-world septic water conditions negatively impacts larvicide efficacy. Larvicide susceptibility and efficacy in septic-water were measured using the larvicides *Bacillus sphaericus*, spinosad, and temephos. *Culex quinquefasciatus* populations were sampled from sites in three Parishes where frequencies of insecticide applications varied, and frequencies of resistance and efficacy were measured relative to a susceptible reference colony. Five-fold resistance to the organophosphate temephos was detected at one site in East Baton Rouge Parish in the spring of 2016, which increased to ten-fold resistance by the end of the mosquito season. Activities of esterases were found to be elevated in wild, temephos-resistant mosquitoes, indicating the potential role of these enzymes as a mechanism of resistance. Water quality did not appear to play a significant role in the efficacy of the larvicides used in this study. The results of this study provide a baseline of comparison for future measurements of susceptibility in *Cx. quinquefasciatus* in Louisiana, and may help inform local mosquito control districts as to the effectiveness and sustainability of their insecticide programs.

CHAPTER 1: LITERATURE REVIEW

1.1 Vector Biology

Mosquitoes are among the deadliest and most debilitating animals on Earth. Anopheline mosquitoes, including *Anopheles gambiae*, the most well studied and primary vector of *Plasmodium falciparum* in Africa (Holt et al. 2002), are largely responsible for the spread of the malarial parasite (Baird 2000). Whereas deaths are trending downward, in 2010 alone, there were still 1,238,000 deaths as a result of malaria (Murray et al. 2012). The malarial parasite may be the most well known mosquito vectored agent of disease, yet there are many more mosquito-borne parasites and pathogens of importance to humans. Dengue virus (Bhatt et al. 2013), yellow fever virus (Mackenzie et al. 2004), chikungunya virus (Pialoux et al. 2007), Zika virus (Hamel et al. 2015), West Nile virus (Nemeth et al. 2011), and the parasite that causes lymphatic filariasis (Ottesen 2006) are among the mosquito vectored parasites and pathogens that cause great suffering to human populations across the world.

West Nile virus is a flavivirus that primarily infects birds, and can be spread to humans by mosquito vectors. Since its arrival in the United States in 1999, West Nile virus has been reported to the Centers for Disease Control approximately 40,000 times, 20,000 of which were neuroinvasive cases, including nearly 2,000 deaths (CDC 2016). Most people infected by West Nile virus experience no symptoms, but some will develop flu like aches and rashes (Sejvar et al. 2003). Neurological complications may arise as a result of West Nile virus infection, with a small percentage of individuals experiencing meningitis, encephalitis, acute flaccid paralysis, or even death (Sejvar et al. 2003). While West Nile virus can cause disease in humans, it is primarily an avian pathogen. Up to 50% of crows

and ravens infected with West Nile virus die as a result of the disease, with symptoms including decreased mobility and diminished reflexes (Nemeth et al. 2011). Mosquitoes from the *Culex* genera are highly susceptible to infection with West Nile virus (Brinton 2002), and are capable of spreading the pathogen to humans and horses, which act as incidental and dead-end hosts (Campbell et al. 2002).

The biology and life history of *Culex* mosquitoes informs their close association with humans, and their prevalence in populated areas around the globe. *Culex quinquefasciatus* Say, the southern house mosquito, is a member of the *Culex pipiens* complex, and is closely related to and shares many traits and behaviors with *Cx. pipiens*, the northern house mosquito. *Culex quinquefasciatus* prefers subtropical climates, and can be found in the southern United States. Conversely, *Cx. pipiens* tend to prefer cooler climates as in the northern United States (Farajollahi et al. 2011). *Culex* mosquitoes are commonly observed entering human dwellings, giving rise to the common name “house mosquito” for *Cx. quinquefasciatus* and *Cx. pipiens* (Reisen 2012). Although some members of the *Cx. pipiens* complex have been shown to be capable of bloodless, autogenous egg development (Strickman and Fonseca 2012), *Cx. quinquefasciatus* is an anautogenous mosquito, requiring blood meals for the development of eggs for each gonotrophic cycle. *Culex quinquefasciatus* females prefer to oviposit in dirty, sediment rich, human-made water, most notably above- and below-ground waste water systems (Reisen 2012). Gravid mosquitoes lay their eggs in floating “rafts” consisting of up to 150 eggs (Roberts and Kokkinn 2010), depending on blood meal source and female age. Development rates of *Cx. quinquefasciatus* are largely determined by temperature, with warmer temperatures decreasing time to adulthood (Rueda et al. 1990). Eggs generally hatch within two days of

oviposition, with larvae progressing through four instars over the course of approximately ten days. During larval instars, these mosquitoes feed upon microorganisms and detritus suspended in the water column, including dust, bacteria, unicellular algae, and small protozoans (Merritt et al. 1992). Following a brief pupal stage, adult males emerge from the water first, followed shortly thereafter by females. Both male and female mosquitoes require sugar intake following eclosion to obtain energy necessary for flight, to help develop gametes, and to mate (Foster 1995). After mating, female *Culex* mosquitoes seek out blood, and undergo a gonotrophic cycle lasting approximately two to three days (Elizondo-Quiroga et al. 2006). Gravid females seek out acceptable water sources to oviposit, and the cycle begins again.

Culex mosquitoes are of great importance in the spread of arboviruses in North America. As part of their gonotrophic cycle, female *Cx. quinquefasciatus* take blood meals from nesting birds and mammals between sundown and sunrise (Farajollahi et al. 2011). Considering birds make up a large part of the blood diet for *Cx. quinquefasciatus*, female mosquitoes occasionally encounter blood infected with avian parasites and pathogens (Fonseca et al. 2004). Many avian parasites and pathogens are known to be vectored by *Culex* mosquitoes, including West Nile virus, St. Louis encephalitis virus, and the avian malaria parasite (Atkinson et al. 2000, Mackenzie et al. 2004, Farajollahi et al. 2011). Since its arrival in Hawaii, *Plasmodium relictum*, also known as the Avian malaria parasite, has worked in tandem with invasive *Cx. quinquefasciatus* and has devastated native bird species (Atkinson et al. 2000). Some arboviruses, including West Nile virus, are of importance to humans because they can cause human disease (Campbell et al. 2002). West Nile virus was first detected in Louisiana in 2002 (Godsey et al. 2005), and is active year-

round in the state (Tesh et al. 2004). Studies have shown that *Culex* mosquitoes in Louisiana primarily feed upon dogs, but passerine birds and humans also make up a significant amount of their blood meal (Niebylski and Meek 1992).

1.2 Mosquito Control

Minimizing the spread of mosquito-vectored pathogens is often performed by controlling mosquito populations rather than directly targeting arboviruses. Mosquito control can be achieved in numerous ways. Adults can be lured away (Okumu et al. 2010), trapped (Reiter 1983), sprayed with adult insecticides (adulticides) (Farajollahi et al. 2012), or deterred from biting by utilization of repellants (Katz et al. 2008). Larvae can be treated with a variety of larval insecticides (larvicides) (Marina et al. 2014), surface-tension breaking oils/films (Corbet et al. 2000), or managed through source-water reduction (Rose 2001).

Local mosquito control districts have operated in southern Louisiana for decades as a result of the large populations of mosquitoes found in its swampy environments and warm climate. Mosquito control was first established in East Baton Rouge Parish in 1979 (EBRMARC 2016). Funding and coverage greatly increased following the arrival of West Nile virus in East Baton Rouge Parish in 2002 (Godsey et al. 2005). Today, East Baton Rouge Mosquito Abatement and Rodent Control workers monitor and treat mosquito populations year round in the hopes of reducing both nuisance and pathogen-carrying mosquitoes. Adulticiding and larviciding are two essential strategies employed in the control of mosquitoes in East Baton Rouge Parish. Adulticiding involves spraying low volumes of insecticides by trucks and airplanes/helicopters to be carried by the wind toward adult mosquitoes. Common adulticides used in East Baton Rouge include:

resmethrin (Scourge™) and prallethrin/sumithrin (Duet®), which are pyrethroids sprayed by truck; and naled (Dibrom™), an organophosphate sprayed by airplane. Less common adulticides, used only occasionally, include permethrin (Aqua-Pursuit™) and deltamethrin (DeltaGard®), which are sprayed by truck. Larvicides are used to treat water sources containing mosquito larvae with water-soluble insecticide formulations. Common larvicides used in East Baton Rouge include: methoprene (Altosid™), a synthetic growth hormone; surface contact oils and films (CocoBear™); spinosad (Natular™), a bacterial metabolite derived from *Saccharopolyspora spinosa*; *Bacillus thuringiensis* subspecies *israelensis* (VectoBac®), containing toxic spores from the bacterium; and *Bacillus sphaericus* (VectoLex™), a bacterium that produces toxic bacterial metabolites.

When chemical control of mosquitoes is required, larvicides are often the first line of defense (Marcombe et al. 2014). Larviciding is a preemptive strategy that can destroy potential vectors of disease before they are capable of spreading (Mains et al. 2015). Many classes of larvicide are used in mosquito management today. The most common larvicides used in the United States are the biorational bacterial agents *Bacillus thuringiensis* subspecies *israelensis* (Lacey 2007) and *Bacillus sphaericus* (Ben-Dov 2014). Spinosad is an effective and recent addition to larvicide strategies, having only received a label for mosquito larvicide purposes in the United States in 2007 (Hertlein et al. 2010). Finally, the organophosphate temephos is a cheaply available larvicide used around the world (Rose 2001). How these larvicides are formulated and used in aquatic environments is essential in understanding potential issues that could arise in the management of mosquitoes.

Whether or not insecticides function is heavily impacted by the medium in which they are suspended. Submergence in water hinders the residual activity of the most

commonly used larvicide, *Bacillus thuringiensis* subspecies *israelensis*, and many attempts have been made to formulate a longer lasting product (Ben-Dov 2014). *Bacillus sphaericus* has greater persistence in polluted, man-made water than *Bacillus thuringiensis* subspecies *israelensis*, which has made it an essential tool in combatting *Culex* mosquitoes (Berry 2012). The degradation of aqueous formulations of spinosad through hydrolysis was shown to be minimal, although partitioning of spinosad onto organic matter and soluble sediments within water remained a concern in one study (Cleveland et al. 2002). The larvicidal organophosphate temephos is poorly soluble in water, tending to migrate to the water surface upon contact (Lacorte et al. 1996). These issues are all present before larvicides encounter a mosquito larva.

Bacillus sphaericus is a spore-forming bacterial agent particularly effective against *Culex* mosquitoes (Baumann et al. 1991). *Bacillus sphaericus* formulations are spread upon bodies of water that are often visited by *Culex* mosquitoes. After dispersal in the water, ingestion of the metabolite by a mosquito larva is followed by *Bacillus sphaericus* pro-toxin activation by the insect's own gut alkalinity (Baumann et al. 1991). Activation of the pro-toxin leads to the release of two crystalline protein toxins, both of which bind to the mosquito's midgut epithelium. The precise mechanism of action of *Bacillus sphaericus* is still not well understood, although mitochondrial swelling and vacuole formation is thought to assist in pore formation in the digestive tract, leading to sepsis and eventual death (Berry 2012). Some formulations of *Bacillus sphaericus* have been shown to persist in the environment for months (Lacey 2007). The long-lasting persistence of *Bacillus sphaericus* in environments is beneficial for short-term mosquito control, but long-release

insecticides have been shown to place populations under strong selective pressure for the development of resistance (Dame et al. 1998).

Spinosad is the active ingredient in a relatively new class of insecticides, the allosteric nicotinic acetylcholine receptor modulators, and has only in the last few years been labeled for use as a mosquito larvicide (Hertlein et al. 2010). Spinosad is a mixture of spinosyn A and spinosyn D, two metabolites of the naturally occurring *Saccharopolyspora spinosa* bacteria. The spinosyns reach their target sites in the nervous system of insects by both cuticular absorption as well as ingestion (Jiang and Mulla 2009). Upon reaching the nervous system, the spinosyns bind at sites that interact with nicotinic acetylcholine and GABA receptors (Salgado 1998). Following binding, neurotransmission is severely impacted, and over-excitation of the nervous system occurs (Salgado 1998), which leads to paralysis, and eventual death. Concerns over the specificity of spinosad have arisen, indicating significant non-target mortality of Odonates, Ephemeroptera, Coleoptera, and Hemiptera at field application rates (Jones and Ottea 2013, Lawler and Dritz 2013, Marina et al. 2014). Louisiana mosquito control districts have slowly begun including spinosad in their arsenal of larvicides.

Temephos, an organophosphate, is a prominent and cheap mosquito larvicide used around the world. As a result of its low price of production (Rose 2001), temephos has been used extensively for decades. Organophosphates act upon the nervous system, where they covalently bond with the active site of acetylcholinesterase (Bajgar 2004). These bonds prevent normal breakdown of the neurotransmitter acetylcholine, culminating in over-excitation of neurons, and death by paralysis (Bajgar 2004). The use of organophosphates has been associated with various acute and chronic effects in non-target

animals, as demonstrated in fish (Hurst et al. 2007) and even humans (Namba 1971). It is important to stress that the risk of human organophosphate exposure at concentrations used by mosquito control is lower than the risk of arbovirus symptoms as a result of not spraying (Peterson et al. 2006).

Despite the popularity of temephos around the world, in the United States, manufacturers of products with temephos as an active ingredient purposefully declined to renew labels with the Environmental Protection Agency beginning in 2011 (EPA 2011). This does not prevent temephos acquired before 2011 from being used; however, nonrenewal has effectively ended future sale and use of temephos in the United States.

1.3 Insecticide Resistance

Insecticide resistance is an inevitable roadblock in any chemical pest control strategy. Resistance has developed when an insect survives a dose of insecticide that normally would have killed it (Hemingway et al. 2002). The first published case of insecticide resistance occurred in 1914 in response to reduced efficacy of sulphur-lime treatment on scale insects (Melander 1914). The earliest case of *Cx. quinquefasciatus* resistance in the United States was detected in 1952 in response to heavy applications of DDT (Gjullin and Isaak 1957). Changes in susceptibility of an insect population, or sensitivity toward an insecticide, can be indicative of the development of resistance, and can be monitored through biological assay (Hoskins and Craig 1962). The World Health Organization has developed commonly used protocols for monitoring larvicide susceptibility through larval mosquito biological assays (WHO 2005). Insecticide resistance has been detected across Louisiana in *Cx. quinquefasciatus* to multiple

insecticides, including fyfanon (Meek and Meisch 1997), resmethrin, and naled (Gordon and Ottea 2012).

Resistance toward *Bacillus sphaericus* limits control options for *Culex* species, and has been documented to exist around the world. Resistance to *Bacillus sphaericus* has been demonstrated in the field in Thailand (Su and Mulla 2004), India (Adak et al. 1995, Rao et al. 1995), and Brazil (Silvafilha et al. 1995). Potential mechanisms underlying *Bacillus sphaericus* resistance include mutations in genes encoding the toxin binding sites, or reductions in the number of total binding sites (Rodcharoen and Mulla 1996). Resistance has developed in as few as 20 generations in both lab and field populations of *Cx. quinquefasciatus* (Rodcharoen and Mulla 1994). Concentrations of *Bacillus sphaericus* that kill 50% of susceptible *Cx. quinquefasciatus* populations (LC_{50}) range from 0.12 ppb (Paul et al. 2005) to 16 ppb (de Melo et al. 2009). Wide ranges in susceptibility, as is the case with *Bacillus sphaericus*, are not entirely unexpected when bioassays of this nature are performed in different labs using different susceptible populations of the same species (Hong et al. 1988).

Spinosad has yet to be heavily incorporated in mosquito larvicide programs, but resistance has been observed and studied in the lab (Su and Cheng 2014a), and resistance has developed in the field with regards to other insect orders (Zhao et al. 2002). A genetic link underlying spinosad resistance was demonstrated in the diamondback moth, and consisted of a homozygous recessive mutation in one allele (Zhao et al. 2002). However, mechanisms of resistance toward spinosad are still under investigation, particularly in mosquitoes. Susceptible LC_{50} values range from 38 ppb (Jones and Ottea 2013) to 100 ppb

(Liu et al. 2004) for susceptible lab colonies, and up to 670 ppb (Su and Cheng 2014a) for field collected naïve populations.

As a result of its low price of production (Rose 2001), temephos has been used extensively for decades, which has likely contributed to the development of resistance in parts of Asia (Peiris and Hemingway 1993, Ali et al. 1999, Cui et al. 2006) and Central America (Wirth and Georghiou 1999, Rodriguez et al. 2001, Bisset et al. 2011). Temephos use is not limited to these regions, however, as it has also been used in Europe (Wirth and Georghiou 1996), South America (Melo-Santos et al. 2010), and in some parts of the United States (Marcombe et al. 2014). Temephos' ability to quickly and effectively kill mosquito larvae, in addition to its cheap cost to manufacture, likely contributed to the extensive development of resistance to it across the world. Temephos resistance has been linked to increased esterase activity (Rodriguez et al. 2001), as well as gene amplification promoting mixed function oxidase and glutathione S-transferase translation (Melo-Santos et al. 2010). Susceptible LC_{50} values range from 0.24 ppb (Ali et al. 1999) to 5.0 ppb (Su and Cheng 2014b).

1.4 Mechanisms of Resistance

Many mechanisms underlie insecticide resistance in mosquitoes, some of which have already been mentioned. Three major mechanisms of resistance in insects include: reduced cuticular penetration (Roberts and Andre 1994), target site modification, and enzymatic detoxication (Li et al. 2007). Cuticular penetration of an insecticide is necessary for some of the most commonly utilized insecticides to reach their target site (Georghiou 1994). Specific mutations which give rise to reduced cuticular penetration are still largely unknown, although associations between reduced penetration and low-level insecticide

resistance have been observed in mosquitoes (Roberts and Andre 1994) and other insects (Ottea et al. 2000). Target site modifications generally involve mutations at the insecticide binding site, leading to the inability of the xenobiotic to bind and function (Georghiou 1994). Single point mutations of the acetylcholinesterase binding site are common mechanisms of organophosphate resistance in mosquitoes, with multiple point mutations conferring greater levels of resistance (Hemingway et al. 2004).

Amplified enzymatic detoxication and excretion of insecticides is a common mechanism behind insecticide resistance in mosquitoes. Ester-detoxifying enzymes (esterases) are split into multiple classes, the most important of which for insecticide purposes are cholinesterases; enzymes that hydrolyze the neurotransmitter acetylcholine (Gomori 1953). These and other esterases naturally act on multiple ester-containing biotic substrates, yet they are also capable of detoxifying xenobiotics that interact at similar target sites and contain esters, including many organophosphates, carbamates, and pyrethroids (Li et al. 2007). Enhanced levels of esterases have been linked to organophosphate and pyrethroid resistance in *Cx. quinquefasciatus* (Peiris and Hemingway 1993, Wirth and Georghiou 1999, Gordon and Ottea 2012), *Cx. pipiens* (Wirth and Georghiou 1996, Cui et al. 2006), and non-Dipteran insect orders (Georghiou 1994). Colorimetric assays can quantify and allow measurement of esterase activity, and are largely based on methods established by van Asperen (1962) and Gomori (1953).

Mosquito control districts and researchers in Louisiana have repeatedly quantified adulticide efficacy and resistance; less research has been devoted toward larvicides. The specific aims of this project include: determining the frequency and intensity of larvicide resistance in *Cx. quinquefasciatus* in southern Louisiana to *Bacillus sphaericus*, spinosad,

and temephos; to examine changes in insecticide susceptibility before and after the mosquito season; to evaluate potential mechanisms of resistance encountered in the field; and to examine the effect of septic water parameters on larvicide efficacy.

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CHAPTER 2: LARVICIDE RESISTANCE

2.1 Introduction

Mosquitoes are among the deadliest and most debilitating animals on Earth. West Nile virus, which is transmitted by *Culex* mosquitoes, can cause severe debilitation and death in humans (Campbell et al. 2002). Over 43,000 human cases of West Nile virus have been reported to the Centers for Disease Control since the virus' arrival in the United States in 1999. Of those cases, 20,000 patients had neurological complications including meningitis, encephalitis, or acute flaccid paralysis (CDC 2016). Nearly 2,000 Americans infected with West Nile virus have died from complications of the disease since 1999, with many more facing symptoms including lifelong disorientation, seizures, and partial paralysis (Sejvar et al. 2003).

Culex quinquefasciatus Say (Diptera: Culicidae), the southern house mosquito, is the most important vector of West Nile virus in the southern United States. This species is a peridomestic mosquito that prefers to oviposit in sediment rich, human-made water, such as above and below ground waste water systems (Reisen 2012). As part of their gonotrophic cycle, female *Cx. quinquefasciatus* take blood meals from nesting birds and mammals between sundown and sunrise. Considering birds make up a large part of the blood diet for *Cx. quinquefasciatus*, female mosquitoes occasionally encounter blood infected with avian viruses. Some of these viruses, including West Nile virus, are especially important because they can cause human disease.

In an effort to minimize the incidence of West Nile virus, mosquito control often employs chemical control strategies towards mosquito vectors using insecticides.

Adulticiding and larviciding are two essential strategies employed in the control of mosquitoes. Adulticiding involves spraying low volumes of insecticides by trucks and airplanes to be carried by the wind toward adult mosquitoes. Common adulticides include pyrethroids sprayed by truck and organophosphates sprayed by airplane. Larvicides are used to treat water sources containing mosquito larvae with water-soluble insecticide formulations. The most commonly used larvicides include bacterial metabolites, synthetic growth hormones, and surface contact oils.

The development of insecticide resistance is a serious concern in all insect pest management strategies, including the control of mosquitoes. Insecticide resistance has developed when an insect survives a dose of insecticide that normally would have killed it (Hemingway et al. 2002). Larvicides are often used as a first line of defense in mosquito control (Marcombe et al. 2014), serving as a preemptive strategy that can destroy potential vectors of disease before they are capable of spreading. Larvicides may be attractive to mosquito control districts due to the ability to rotate between different products, which can assist in curbing the development of resistance (Georghiou 1994). Many larvicides are provided in slow release formulations, which allows for residual insecticidal effects. However, slow release insecticides have been demonstrated to place populations under high selective pressure for multiple generations, increasing the rate at which resistance develops (Dame et al. 1998). Considering the majority of a mosquito control district's chemical control efforts are often performed using larvicides, understanding the local status of larvicide resistance can be pivotal.

Mosquito control was first established in our study area of East Baton Rouge Parish in 1979 (EBRMARC 2016). In the present study, we hypothesized that decades of larvicide

treatment in East Baton Rouge Parish resulted in the development of resistance in *Cx. quinquefasciatus*. This hypothesis was tested by collecting *Cx. quinquefasciatus* from across Louisiana and performing biological assays that examined larvicide susceptibility. When resistance was encountered in the wild, further investigation into xenobiotic metabolism was performed to evaluate potential mechanisms of resistance. The results of this study may help inform local mosquito control districts as to potential strengths and shortcomings of their larvicide treatment strategy.

2.2 Materials and Methods

2.2.1 Chemicals

Sodium phosphate (monobasic monohydrate (>98%) + sodium phosphate dibasic heptahydrate (98%)), Brilliant Blue G-250, Fast Blue B salt (approx. 95%), 1-naphthyl acetate (α -NA) (>98%), and technical grade spinosad (65.56% spinosyn A, 31.37% spinosyn D; 96.9%) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (99.7%), sodium hydroxide (ACS grade), and sodium dodecyl sulfate (SDS) (99%) were purchased from Fisher Scientific (Kansas City, MO). Bovine serum albumin (biotechnology grade) and acetone (pesticide grade) were purchased from Amresco (Solon, OH). Ethyl alcohol (200 proof) was purchased from Pharmco-Aaper (Brookfield, CT). Formulations of *Bacillus sphaericus* 2362 (VectoLex® WDG, 51.2%) and temephos (ABATE® 4-E, (O,O,O,O-Tetramethyl O,O-sulfanediylbis(1,4-phenylene) diphosphorothioate), 44.6%) were donated by East Baton Rouge Mosquito Abatement and Rodent Control (Baton Rouge, LA). Lactalbumin and brewer's yeast were donated by the Livingston Parish Mosquito Abatement District (Denham Springs, LA). Fish fertilizer (Alaska® 5-1-1 Fish Fertilizer) was purchased locally.

2.2.2 Insects

East Baton Rouge Mosquito Abatement and Rodent Control provided a susceptible, reference strain of *Cx. quinquefasciatus* (henceforth Sebring) to the Louisiana State University medical entomology lab in January of 2015 as egg rafts. This colony has since been maintained in the medical entomology insectary within the Life Sciences building on the Louisiana State University campus. The Sebring strain originates from Sebring, FL and was originally collected and maintained by the local USDA Agricultural Research Station (Stancil 2000).

Egg rafts of *Cx. quinquefasciatus* were transferred to white plastic trays (25 x 35 cm) filled with 1.5 liters of diH₂O that had been exposed to open air for ≥24 h. Trays were incubated at 27°C (±2) and a 14h light:10h dark cycle. A mixture of 75 mg lactalbumin and 75 mg brewer's yeast was sprinkled on to the water surface every weekday for food. Following pupation, pupae were removed individually by pipetting, and transferred to shallow glass cups that were then placed in collapsible cages (31cm³) inside the medical entomology insectary, set to 27°C (±2) and 14h light:10h dark.

Adult mosquitoes were provided 10% sugar solution *ad libitum* in 150 ml Erlenmeyer flasks with cotton wicks. Sugar water was refreshed twice weekly, and wicks changed weekly. Cages containing adults were draped in damp cotton cloth and shrouded partially by plastic bags in order to increase humidity. A Hobo® (Onset Computer Corporation, Bourne MA) sensor placed near adult cages monitored temperature and light cycle. Sugar was removed from cages one day prior to blood feeding, after which defibrinated chicken-blood (Rockland™ Immunochemicals, Limerick, PA) was provided for ≥2 hours, using an artificial feeder (Hemotek® Ltd, England) with stretched Parafilm M®

(Bemis Company, Oshkosh, WI) as a membrane. Following blood feeding, a shallow black cup containing aged diH₂O was inserted into the cage for oviposition. Egg rafts were later removed from the cups to white plastic trays.

2.2.3 Mosquito Sampling Sites

Eight sampling sites were chosen to monitor larvicide susceptibility: four in East Baton Rouge Parish, three in Livingston Parish, and one in St. Helena Parish (Figure 1).

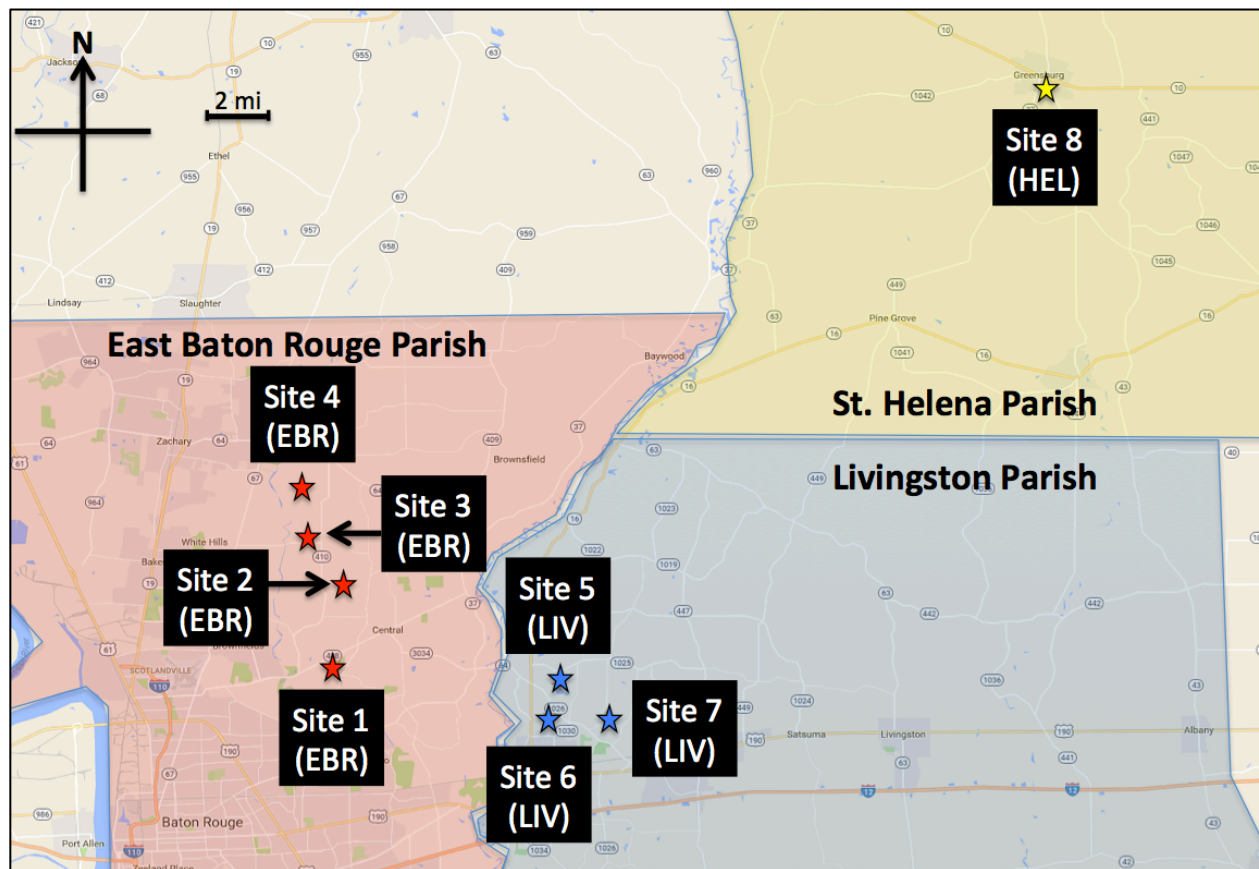


Fig. 1. Map of mosquito sampling sites in East Baton Rouge, Livingston, and St. Helena Parishes.

Sites in East Baton Rouge Parish were chosen with the assistance of East Baton Rouge Mosquito Abatement and Rodent Control, and were located in areas that received consistently high numbers of larvicide treatments. Sites in Livingston Parish were chosen with the assistance of a former Livingston Parish Mosquito Abatement District employee,

and were located in areas that received consistently high numbers of larvicide treatments prior to the District's closure in 2013. The site in St. Helena Parish was chosen with the assistance of the local LSU AgCenter extension office, and was located in an area of high mosquito density, with no known history of larvicide application.

Egg rafts were collected by placement of gravid traps in shaded areas along roadside septic ditches. Gravid traps consisted of a black plastic container (50x40x18cm) loaded with a gravid water mixture (4 liters of diH₂O and 50 ml of fish fertilizer). Gravid water was changed weekly, and egg raft collections were made twice per week.

Populations of wild mosquito were collected in spring 2016 from East Baton Rouge Parish sites between 31 March and 19 April, and again in the fall between 21 October and 15 November. Sites within Livingston Parish were sampled from 29 April and 12 May. The St. Helena Parish site was sampled from 13 May and 27 May. East Baton Rouge Mosquito Abatement and Rodent Control recorded larvicide (Table 1) and adulticide (Table 2) treatments and frequencies at sites 1 - 3 in East Baton Rouge Parish during the mosquito season. All insecticide treatments by mosquito control were performed following label instructions at maximum label rates.

Table 1. Number and type of larvicide treatments at sites in East Baton Rouge Parish between 31 March and 15 November 2016.

	VectoBac® <i>(Bacillus thuringiensis israelensis)</i>	VectoLex® <i>(Bacillus sphaericus)</i>	Total
Site 1	13	13	26
Site 2	6	3	9
Site 3	14	16	30
Total	33	32	65

To ensure sampled mosquitoes were *Cx. quinquefasciatus*, sub-samples containing approximately 20 late-3rd and/or early-4th instars were taken from hatched egg rafts of sample sites and identified to species using a dichotomous key (Burkett-Cadena 2013). Samples containing any individuals not identified as *Cx. quinquefasciatus* were discarded.

Table 2. Number and type of adulticide treatments at sites in East Baton Rouge Parish between 31 March and 15 November 2016.

	Aqua-Pursuit™ (Permethrin)	DeltaGard® (Deltamethrin)	Duet® (Prallethrin & Sumithrin)	Scourge® (Resmethrin)	Total
Site 1	1	0	17	1	19
Site 2	3	1	7	26	37
Site 3	0	0	0	26	26
Total	4	1	24	53	82

2.2.4 Biological Assays

Stock solutions (10,000 ppb) of *Bacillus sphaericus* and temephos were diluted in ddH₂O and stored in amber bottles at room temperature. A stock solution (100,000 ppb) of spinosad was diluted in ACS grade acetone and was stored in amber glass at room temperature. Stock solutions were serially diluted with distilled water immediately prior to biological assay.

Initial susceptibilities of the Sebring colony were measured in Pyrex® glass petri dishes (No. 3140-100; 10x5 cm). Prior to bioassay, dishes were washed with soap and diH₂O, rinsed with 0.1 M NaOH, rinsed again with diH₂O, sprayed with bulk acetone, rinsed a final time with diH₂O, and then dried in a heating chamber at 80°C overnight. Comparisons were made between glass and paper containers by performing bioassays again in 200 ml paper cups (Karat® 6oz paper food containers). Considering results did

not differ, paper containers were used for the remainder of this study. Results of glass and paper comparisons are available in the Appendix.

Approximately 20 late-3rd and/or early-4th instar *Cx. quinquefasciatus* were removed from rearing trays with mesh nets, and placed into containers holding 100 ml of diH₂O. Following addition of larvae, larvicides were aliquoted into each cup, giving desired final concentrations. Cups containing 100 ml of diH₂O were used as controls. Plastic wrap was placed over cups to prevent desiccation, and cups incubated at 27°C and a 14h light:10h dark cycle. Mortality was read after 24 hours for spinosad and temephos, and 48 hours for *Bacillus sphaericus*. Larvae were considered dead if unresponsive upon prodding with a sharp pencil tip.

Insecticide susceptibility of larvae from the Sebring colony was measured using triplicate bioassays and at least seven different concentrations per larvicide. Final concentrations of larvicides in the assays ranged from 0.01 – 10 ppb for *Bacillus sphaericus*, 1 – 5,000 ppb for spinosad, and 0.01 – 10 ppb for temephos. Susceptibilities were measured over four to five different determination dates using freshly prepared concentrations of insecticides.

Concentrations of insecticide that killed approximately 99% of reference Sebring mosquitoes (LC₉₉) were determined by probit analysis (Hoskins and Craig 1962) for each larvicide, and were used as diagnostic concentrations to measure resistance frequencies in mosquito populations from experimental sites. For these assays, treatments used 20 larvae (in triplicate) and the LC₉₉ of *Bacillus sphaericus* (9 ppb), spinosad (1,700 ppb), and temephos (4.5 ppb). Susceptibility at this concentration was determined from collections made from at least three separate dates. As a means of comparison, mortality of the Sebring

colony to the reference LC₉₉ was also measured in triplicate over three separate determination dates. Experimental sites that had significantly higher survival than the Sebring colony when exposed to the diagnostic concentration were sampled again, and specimens subjected to a full range of concentrations as appropriate for probit analysis. Resistance ratios were calculated for resistant populations by dividing the measured site LC₅₀ by the LC₅₀ of the Sebring reference colony.

2.2.5 Enzyme Assays

Esterase activities toward α -naphthyl acetate were measured following the assay of Gomori (1953), with modifications by van Asperen (1962) and Grant et. al (1989). *Culex quinquefasciatus* were individually homogenized as adults or fourth instars using 10 strokes of an all glass mortar and pestle containing 500 μ l of sodium phosphate buffer (0.1M, pH 7.4). Homogenates were then centrifuged at 14,400 rpm and 4°C for 10 min. Twenty μ l of supernatant (0.04 insect equivalents) were pipetted in triplicate in a 96-well plate (Fisherbrand® Flat Bottom Non-Sterile Plate), followed by 200 μ l of 0.3 mM α -NA (prepared by mixing 0.2793g of α -NA in 50ml acetone, and then diluting 1 ml of this mixture in 99 ml of 0.1M pH 7.4 phosphate buffer; 0.3 mM final concentration). Controls received 20 μ l of buffer in place of homogenate. Reactions were stopped after 15 minutes by addition of 50 μ l of FastBlue dye (prepared by mixing 0.15g FastBlue B salt in 15 ml diH₂O and 35 ml 5% SDS solution). Optical density was read after five minutes using a SpectraMAX 190® plate reader (Molecular Devices LLC, Sunnyvale CA) at 570 nm and 27°C. Optical density was converted to μ moles min⁻¹ using an experimentally derived extinction coefficient of α -naphthol ($OD=0.0235*[\alpha\text{-naphthol}]-0.0376$; $R^2=0.998$). Protein

in reaction mixtures was determined using the method of Bradford (1976), with bovine serum albumin as the standard.

2.2.6 Statistical Analysis

All statistical analyses were performed using MiniTab® ver 17 (Minitab 2016). Mortality data from the Sebring colony and sites 3 and 8 were subjected to probit analysis, calculating slope, LC₅₀ and LC₉₉ values. One-way ANOVA with *ad hoc* Tukey tests ($\alpha=0.05$) were run to compare differences in mortality following exposure to diagnostic concentrations between the Sebring colony and experimental sites. Student's T-tests were used to compare mortality data from sites 1-3 in the spring and the fall of 2016, and to compare esterase activity between the Sebring colony and specimens collected from site 3.

2.3 Results

2.3.1 Susceptibility to *Bacillus sphaericus*, spinosad, and temephos

The reference Sebring colony was subjected to varied concentrations of larvicides for probit analysis, producing baseline LC₅₀ and LC₉₉ values for *Bacillus sphaericus*, spinosad, and temephos (Figure 2). The Sebring colony was most susceptible to *Bacillus sphaericus* (LC₅₀=0.21ppb), followed by temephos (LC₅₀=0.55ppb), and was least susceptible to spinosad (LC₅₀=50ppb). Comparisons between concentration response lines performed in glass and paper containers are available in the Appendix: Glass/Paper Cup Comparisons.

Bioassays with Sebring and experimental site larvae suggest widespread susceptibility at all sites to *Bacillus sphaericus* and spinosad (Table 3). Resistance frequency at site 6 in Livingston Parish in response to treatment with *Bacillus sphaericus* was relatively high, with 26% of individuals surviving in response to the diagnostic LC₉₉, but

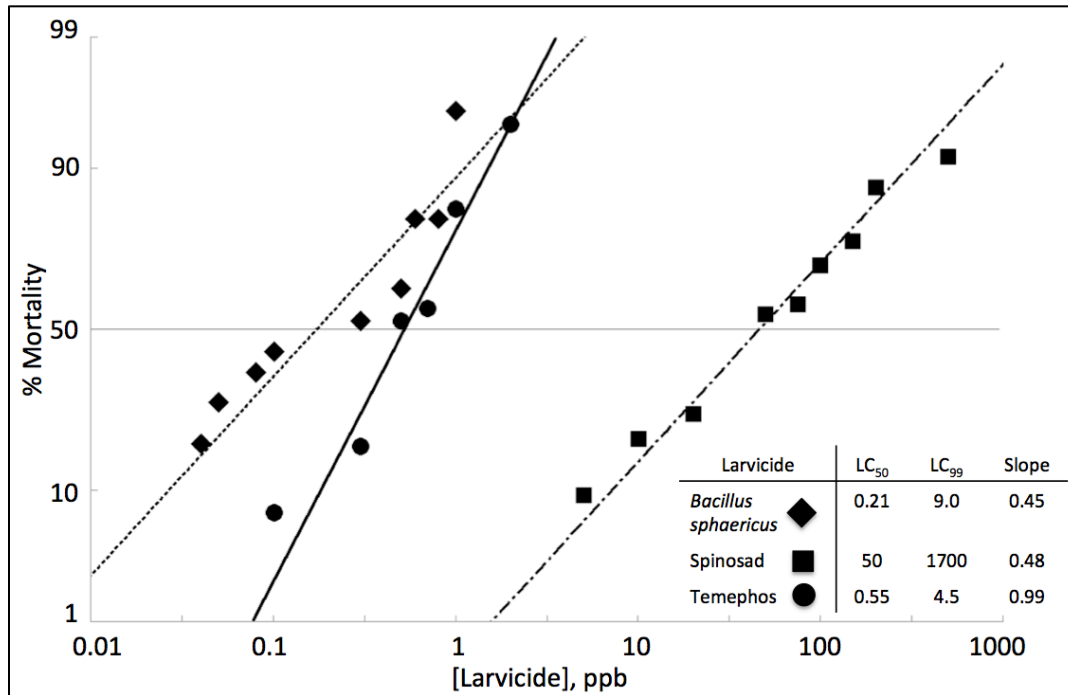


Fig. 2. Susceptibility of larvae from the reference Sebring colony to *Bacillus sphaericus*, spinosad, or temephos. Data were collected from at least 8 concentrations of larvicide, with at least 20 larvae treated in triplicate over 3-5 separate determination dates. LC₅₀ and LC₉₉ were determined using probit analysis.

Table 3. Resistance frequency of the Sebring reference strain and all experimental sites at a diagnostic concentration (susceptible LC₉₉).

Site	Percent Survival (\pm SD)					
	<i>Bacillus sphaericus</i>	n	Spinosad	n	Temephos	n
Sebring (S)	4 (\pm 4.5) ^a	4	1 (\pm 1.0) ^a	4	2 (\pm 1.9) ^a	4
1 (EBR)	16 (\pm 9.3) ^a	3	3 (\pm 2.3) ^a	3	38 (\pm 14) ^{abc}	3
2 (EBR)	13 (\pm 8.2) ^a	3	1 (\pm 1.0) ^a	3	23 (\pm 18) ^{ab}	3
3 (EBR)	12 (\pm 5.1) ^a	4	1 (\pm 1.0) ^a	3	54 (\pm 21) ^{bc}	4
4 (EBR)	17 (\pm 15) ^a	2	6 (\pm 6.4) ^a	2	44 (\pm 6.4) ^{abc}	2
5 (LIV)	11 (\pm 1.5) ^a	3	10 (\pm 10) ^a	3	48 (\pm 10) ^{bc}	3
6 (LIV)	26 (\pm 21) ^a	3	5 (\pm 7.1) ^a	2	76 (\pm 10) ^c	3
7 (LIV)	7 (\pm 2.7) ^a	3	5 (\pm 4.5) ^a	3	54 (\pm 22) ^{bc}	3
8 (HEL)	9 (\pm 3.5) ^a	3	5 (\pm 6.9) ^a	3	31 (\pm 18) ^{ab}	3

Analysis of variance (ANOVA) with Tukey pairwise comparisons ($\alpha=0.05$) for *Bacillus sphaericus* (df=8,19; F=0.57; p=0.199), spinosad (df=8,17; F=1.14; p=0.387), and temephos (df=8,19; F=6.82; p<0.000). Survival at sites with different letters are significantly different from one-another.

this was not statistically significantly different from the Sebring survival response. Survival at the diagnostic concentration was highest and significant ($P\text{-value}<0.000$) in populations treated with temephos. A significantly elevated frequency of resistance compared to the reference Sebring strain was detected in mosquitoes from site 3 in East Baton Rouge Parish, and sites 5, 6, and 7 in Livingston Parish. Site 6 had the highest frequency of resistance, with 76% of individuals surviving the diagnostic concentration, followed by sites 3 and 7 with 54% survival each. Based on probit analysis of mortality measured in bioassays with multiple concentrations of temephos, the resistant population at site 3 was found to have an LC_{50} of 2.6 ppb, and a resistance ratio of 4.7 compared to the reference Sebring colony (Figure 3).

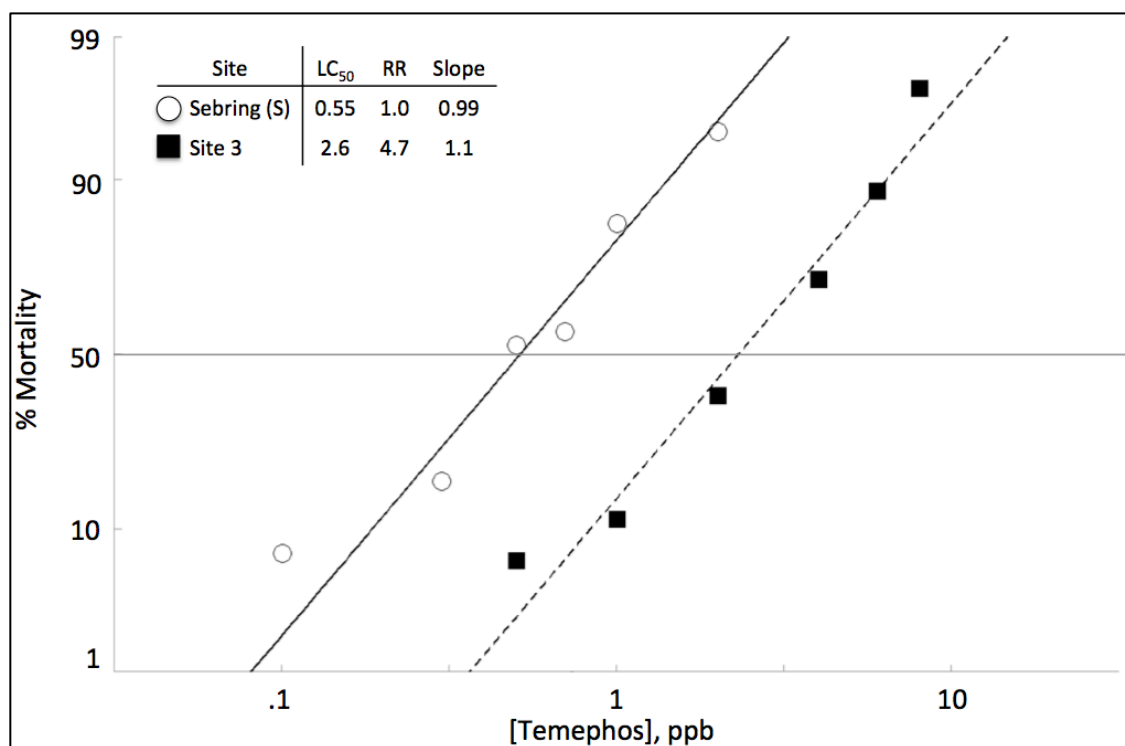


Fig. 3. Susceptibility of the reference Sebring colony and site 3 in response to varied concentrations of temephos. Data were collected from at least 6 concentrations of temephos, with at least 20 mosquitoes treated in triplicate over 3 determination dates. Concentration response line was plotted using probit analysis. Resistance ratio (RR) was calculated by dividing site LC_{50} by Sebring LC_{50} .

Fourth instar *Cx. quinquefasciatus* from site 8 in St. Helena Parish had a resistance frequency of 31% toward temephos when exposed to the diagnostic LC₉₉ (Table 3), which was not statistically significantly different from the reference Sebring response, but was high enough to warrant further analysis. Following additional bioassays of wild mosquitoes from site 8 in St. Helena Parish, we measured a LC₅₀ of 1.4 ppb toward temephos (Figure 4), a 2.5-fold reduction in susceptibility compared to the reference Sebring colony.

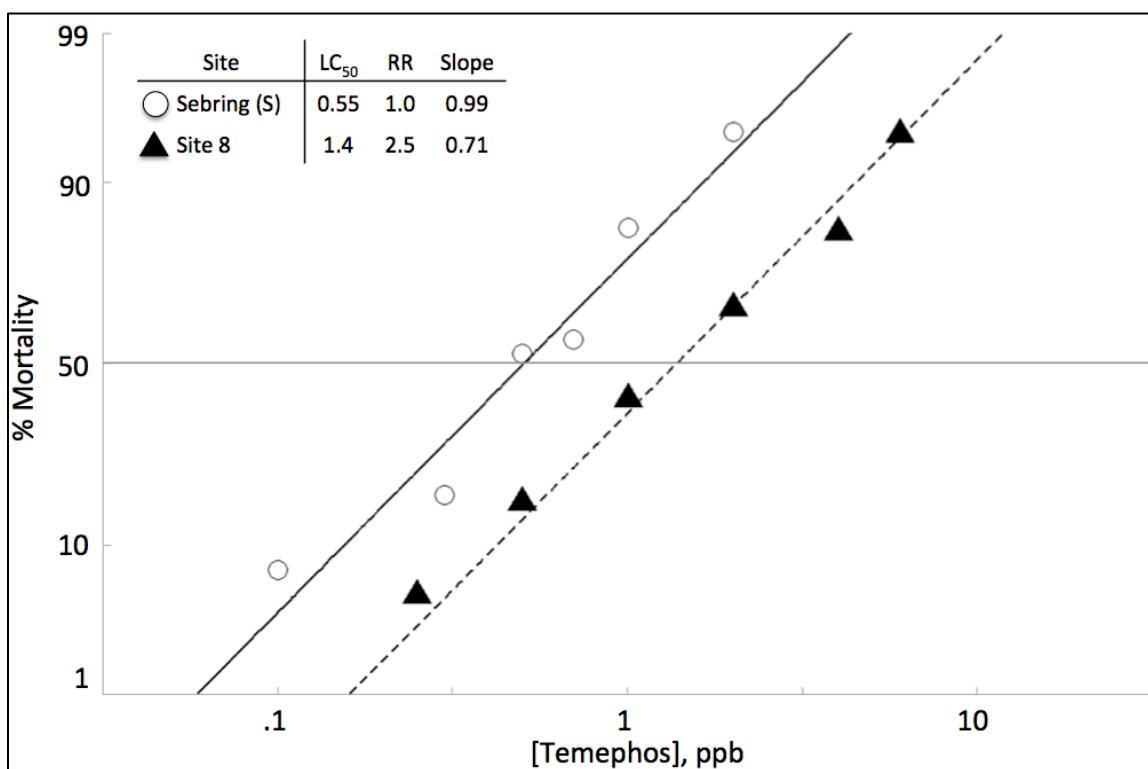


Fig. 4. Susceptibility of the reference Sebring colony and site 8 in St. Helena Parish in response to varied concentrations of temephos. Data were collected from at least 6 concentrations of temephos, with at least 20 mosquitoes treated in triplicate over 3 determination dates. Concentration response line was plotted using probit analysis. Resistance ratio (RR) was calculated by dividing site LC₅₀ by Sebring LC₅₀.

There was no significant change in susceptibility toward *Bacillus sphaericus* and spinosad at sites 1-3 from the beginning to the end of the 2016 mosquito season (Table 4). Susceptibility to temephos increased at all sites. Differences were most dramatic at site 1, where survivorship following exposure to the diagnostic LC₉₉ dropped from 38% in the

Table 4. Percent survival at sites 1, 2, and 3 in East Baton Rouge Parish in the spring and fall of 2016.

Site	Percent Survival (\pm SD)								
	<i>Bacillus sphaericus</i>			Spinosad			Temephos		
	Spring	Fall	P	Spring	Fall	P	Spring	Fall	P
1	16 (\pm 9)	11 (\pm 10)	0.61	3 (\pm 2)	2 (\pm 1)	0.46	38 (\pm 10)	2 (\pm 2)	0.05
2	13 (\pm 8)	2 (\pm 8)	0.15	1 (\pm 1)	2 (\pm 2)	0.57	23 (\pm 20)	7 (\pm 1)	0.26
3	12 (\pm 5)	8 (\pm 8)	0.57	1 (\pm 1)	2 (\pm 3)	0.60	54 (\pm 20)	42 (\pm 20)	0.44

spring to 2% in the fall ($df=2$, T-value=-4.4, P-value=0.05). Further bioassays with temephos and mosquitoes from site 3 resulted in an LC_{50} of 4.6 ppb in the fall (Figure 5), an 8.4-fold decrease in susceptibility compared to the reference Sebring colony. No linear correlations were found between number of insecticide treatments throughout the season by mosquito control (Tables 1 & 2) and frequency of resistance in the fall.

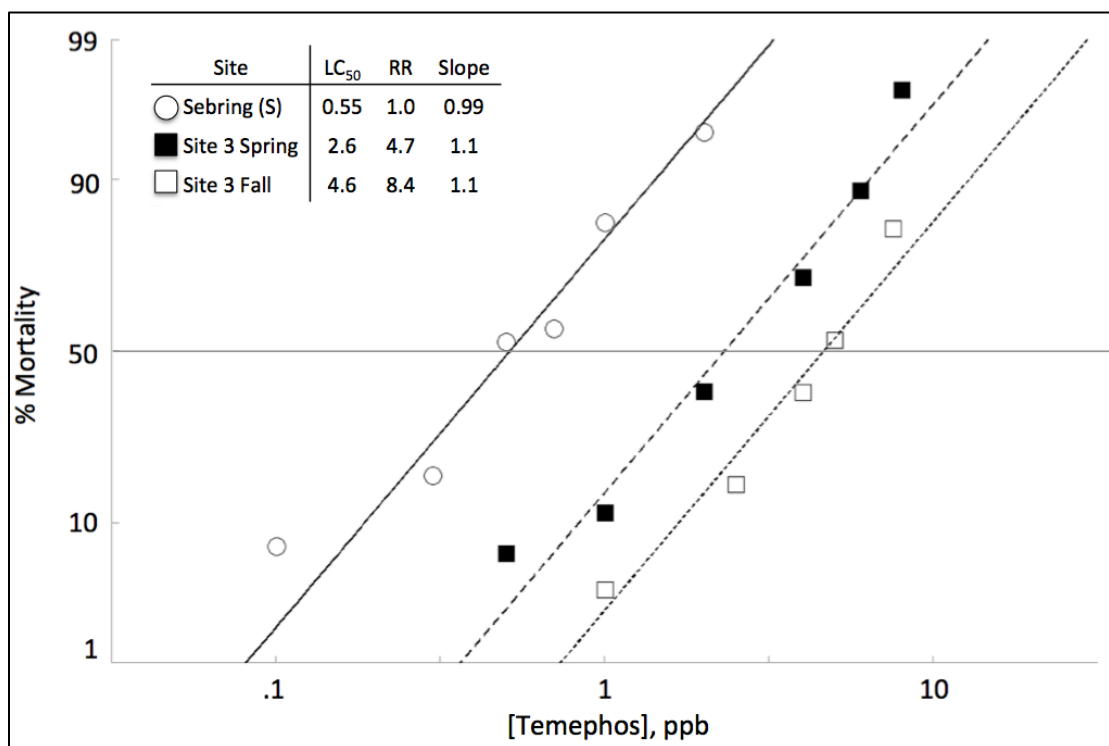


Fig. 5. Susceptibility of the reference Sebring colony, site 3 in spring, and site 3 in fall in response to varied concentrations of temephos. Concentration response line was plotted using probit analysis. Resistance ratio (RR) was calculated by dividing site LC_{50} by Sebring LC_{50} .

2.3.2 Esterase Activity

Esterase activity was significantly higher in larvae from site 3 compared to the Sebring reference colony (Figure 6). The mean esterase activity for Sebring individuals ($272 \mu\text{M min}^{-1}\text{mgprot}^{-1}$) was significantly lower than mosquitoes from site 3 ($770 \mu\text{M min}^{-1}\text{mgprot}^{-1}$). Approximately 17% of larvae from site 3 had esterase activity above 1,000 $\mu\text{M min}^{-1}\text{mgprot}^{-1}$.

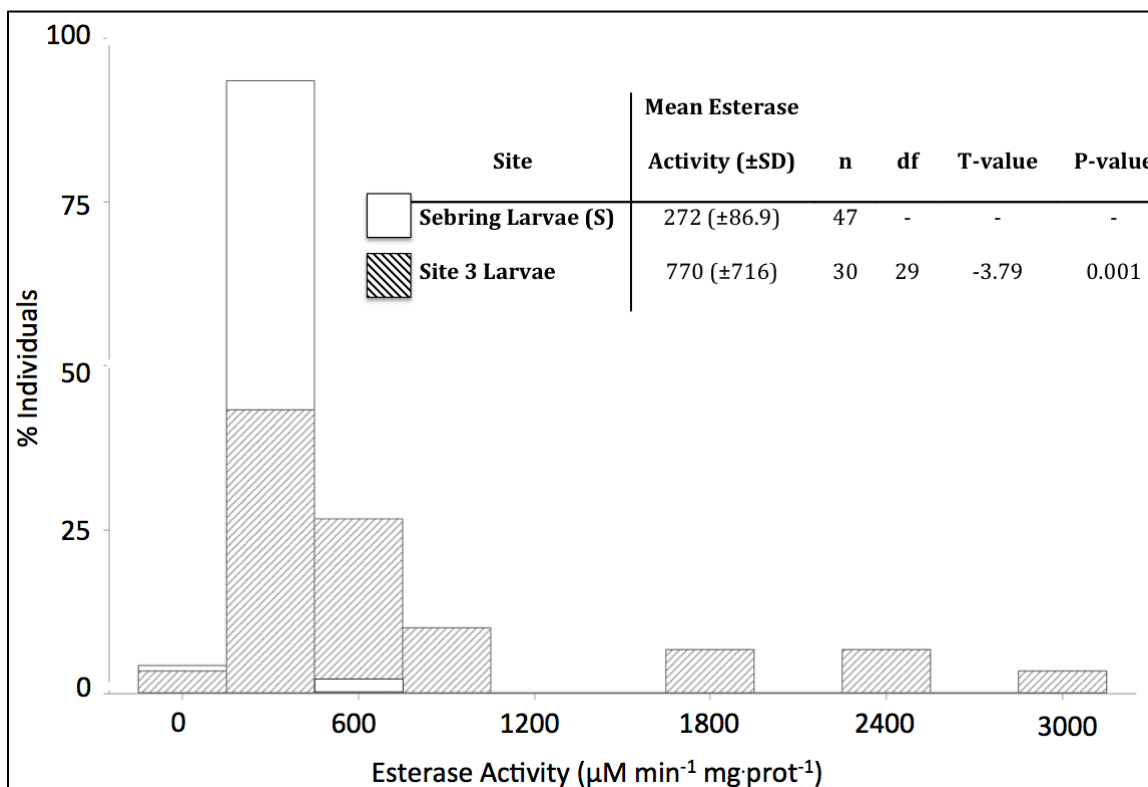


Fig. 6. Esterase activity in reference Sebring larvae and temephos-resistant larvae from site 3 in East Baton Rouge Parish. Student's T-test ($\alpha=0.05$) were performed, with P-values <0.05 indicating significant difference from larvae of the Sebring colony.

Esterase activity was not significantly different between adults from site 3 ($372 \mu\text{M min}^{-1}\text{mgprot}^{-1}$) compared to adults from the Sebring reference colony ($355 \mu\text{M min}^{-1}\text{mgprot}^{-1}$) (Figure 7). However, Sebring adults ($372 \mu\text{M min}^{-1}\text{mgprot}^{-1}$) did have

significantly higher mean esterase activity than Sebring larvae ($272 \mu\text{M min}^{-1}\text{mg:prot}^{-1}$) and site 3 larvae ($272 \mu\text{M min}^{-1}\text{mg:prot}^{-1}$).

2.4 Discussion

Wild *Cx. quinquefasciatus* larvae from all sampling sites were largely susceptible to spinosad. Spinosad belongs to a new class of insecticide with a novel mechanism of action (Hertlein et al. 2010), and to date the only reported case of spinosad resistance in mosquitoes was in a lab colony specifically bred for that purpose (Su and Cheng 2014a). However, considering multiple-resistance to *Bacillus sphaericus* has been detected in larval *Cx. quinquefasciatus* treated with spinosad (Su and Cheng 2014b), concerns existed over whether mosquitoes could be resistant toward spinosad before it was widely adopted in Louisiana. However, resistance does not appear to have developed at sampling sites with regards to spinosad.

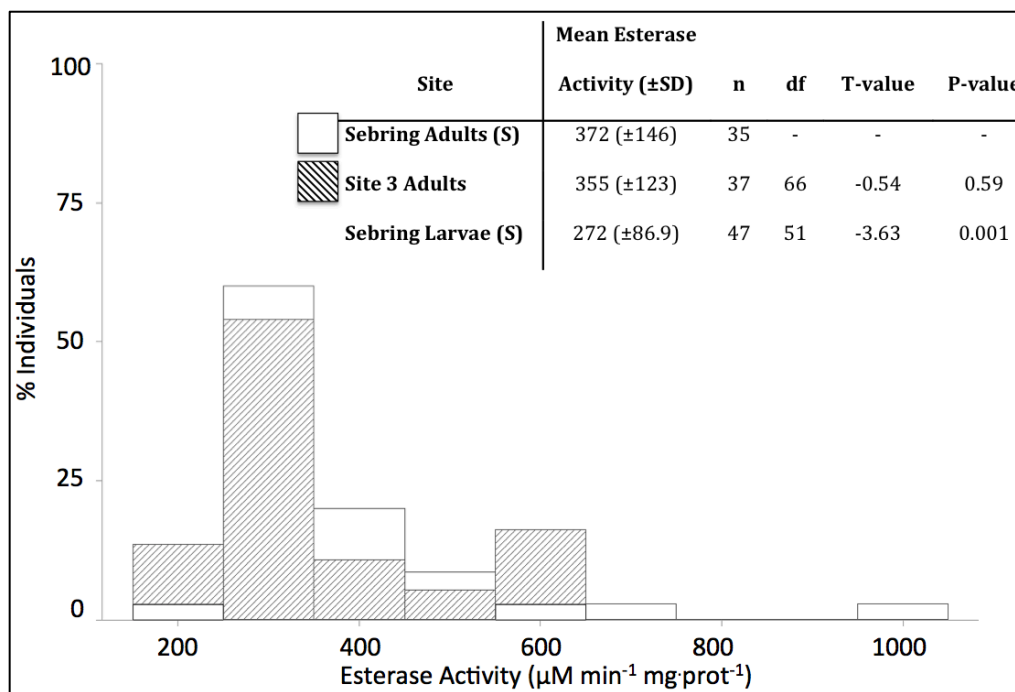


Fig. 7. Esterase activity in reference Sebring adults and temephos-resistant adults from site 3 in East Baton Rouge Parish. P-values <0.05 indicate significant differences from adults of the Sebring colony.

Similarly, larval *Cx. quinquefasciatus* from sampling sites were susceptible to *Bacillus sphaericus*. One site in Livingston Parish had a slightly elevated frequency of resistance (26% survival following treatment with the reference LC₉₉), but this was not statistically significant. The lack of resistant populations in East Baton Rouge Parish is noteworthy given *Bacillus sphaericus* has been used frequently as a larvicide for over a decade at these sites. For example, during the summer of 2016, *Bacillus sphaericus* was sprayed 32 times at sampling sites in Baton Rouge. After a similar number of field treatments with *Bacillus sphaericus* over the course of a mosquito season, researchers in India (Adak et al. 1995) and Brazil (Silvafilha et al. 1995) noticed a reduction in its efficacy, and found a 3- to 10-fold reduction in susceptibility of wild *Cx. quinquefasciatus*. No such reduction in efficacy was determined through bioassay, or observed by East Baton Rouge Mosquito Abatement and Rodent Control workers as communicated to the researchers. The lack of evidence of resistance to *Bacillus sphaericus* at sampling sites is reassuring for the continued use of this product.

Resistance was detected toward temephos at one site in East Baton Rouge Parish, where a resistance frequency of 54% was measured in response to treatment with a diagnostic concentration. The World Health Organization recommends establishing diagnostic concentrations of insecticides to screen for insecticide resistance (WHO 2005), but confirmation and quantification of resistance through biological assays remains invaluable. In this study, further evaluation of resistance using concentration response lines indicated a 4.7-fold decrease in susceptibility in the spring of 2016 compared to the reference Sebring colony, confirming the presence and intensity of resistance. However, the convenience of screening for resistance with diagnostic concentrations may not be an

adequate substitute for resistance evaluation using a full range of insecticide concentrations.

Both frequency and intensity of resistance changed in East Baton Rouge Parish over the course of the 2016 mosquito season. A prior study found frequencies of resistance in *Cx. pipiens* from southern France to increase through periods of heavy insecticide application, followed by recovery of susceptible allele frequencies in the Fall and Winter months (Lenormand et. al 1999). However, this may not be a good indicator of *Culex* populations in southern Louisiana, where mosquito control is necessary year-round due to the warm climate. In the present study, resistance frequencies decreased from spring to fall of 2016 across nearly every site in East Baton Rouge Parish. The frequency of temephos resistance lowered slightly over the summer the site where resistance was first detected, from 54% resistance in the spring to 42% in fall of 2016, though this recovery was not statistically significant. However, evaluation of the intensity of resistance at this site compared to the reference Sebring colony showed an increase over this timespan, from 4.7-fold resistance in the spring, to 8.4-fold in the fall. The use of diagnostic concentrations to screen for resistance is common (Liu et. al 2013, Gordon and Ottea 2012, Tetreau et. al 2013), but this study shows the limitations of that approach. The intensity of temephos resistance nearly doubled at site 3 over the course of the mosquito season, yet the frequency of resistance (as measured by treatment with a diagnostic concentration) remained the same.

The source of temephos resistance in East Baton Rouge Parish could be the result of cross-resistance. Temephos-resistant *Aedes aegypti* were found to be cross-resistant to other organophosphates (Wirth and Georghiou 1999) and the pyrethroid cypermethrin

(Melo-Santos et al. 2010). Researchers that examined the persistence of resistance in all life-stages found *Ae. aegypti* that were resistant to temephos as larvae maintained that resistance into adulthood, and were cross resistant to other organophosphates and pyrethroids (Tikar et al. 2009). These cases indicate the possibility for the development of cross-resistance to other active ingredients as a result of temephos treatment. In this particular case, temephos resistance may have developed as a result of cross-resistance from adulticide treatments with resmethrin. Mosquito control sprayed resmethrin 26 times at the temephos resistant site over the course of the 2016 mosquito season. Amplified esterase activity has been demonstrated to confer cross-resistance between organophosphates and pyrethroids in *Culex* mosquitoes (Strong et al. 2008). Future work examining potential cross-resistance could include performing susceptibility bioassays on adult *Cx. quinquefasciatus* from site 3 with resmethrin, to determine whether a correlation exists between resmethrin and temephos resistance.

Compared to the Sebring reference colony, esterase activity was significantly elevated in *Cx. quinquefasciatus* collected from the temephos-resistant site in Baton Rouge. Esterase activity was highly variable in temephos-resistant mosquitoes, with 17% of the population expressing 10-fold or greater enzyme activity. Elevated esterase activities were shown in *Cx. quinquefasciatus* from East Baton Rouge Parish in 2010, and at that time were correlated with applications of the organophosphate naled (Gordon and Ottea 2012). Resistance to temephos has been linked to amplified esterase activities on multiple occasions (Wirth and Georghiou 1999, Bisset et al. 2011). However, elevated esterase

activity alone is not sufficient evidence for explaining a mechanism of resistance, considering cases where there is no correlation between the two (Harold and Ottea 1997, Gordon and Ottea 2012).

In summary, this study detected temephos resistance in larval *Cx. quinquefasciatus* from East Baton Rouge Parish with a possible link to increased esterase activity. More work is needed to clarify the role of esterases in this resistant population, in addition to examining other potential mechanisms of organophosphate resistance, including non-hydrolytic detoxication as well as potential target site mutations within the acetylcholinesterase enzyme itself. However, more fundamental than investigating potential mechanisms is further examination of the origin of temephos resistance. Performing adult *Cx. quinquefasciatus* susceptibility bioassays with adulticides used in Baton Rouge could help inform whether development of cross resistance between life stages has occurred. Nevertheless, the lack of resistance to *Bacillus sphaericus* and spinosad, larvicides that are used in the Parish, is a positive indicator that larvicide susceptibility has been maintained in Baton Rouge.

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CHAPTER 3: WATER QUALITY

3.1 Introduction

Mosquitoes are among the deadliest and most debilitating animals on Earth. West Nile virus, which is transmitted by *Culex* mosquitoes, can cause severe debilitation and death in humans (Campbell et al. 2002). Over 43,000 human cases of West Nile virus have been reported to the Centers for Disease Control since the virus' arrival in the United States in 1999. Of those cases, 20,000 patients had neurological complications including meningitis, encephalitis, or acute flaccid paralysis (CDC 2016). Nearly 2,000 Americans infected with West Nile virus have died from complications of the disease since 1999, with many more facing symptoms including lifelong disorientation, seizures, and partial paralysis (Sejvar et al. 2003).

Culex quinquefasciatus Say (Diptera: Culicidae), the southern house mosquito, is the most important vector of West Nile virus in the southern United States. This species is a peridomestic mosquito that prefers to oviposit in sediment rich, human-made water, such as above and below ground waste water systems (Reisen 2012). As part of their gonotrophic cycle, female *Cx. quinquefasciatus* take blood meals from nesting birds and mammals between sundown and sunrise. Considering birds make up a large part of the blood diet for *Cx. quinquefasciatus*, female mosquitoes occasionally encounter blood infected with avian viruses. Some of these viruses, including West Nile virus, are especially important because they can cause human disease.

In an effort to minimize the incidence of West Nile virus, mosquito control often employs chemical control strategies towards mosquito vectors using insecticides.

Adulticiding and larviciding are two essential strategies employed in the control of mosquitoes. Adulticiding involves spraying low volumes of insecticides by trucks and airplanes to be carried by the wind toward adult mosquitoes. Common adulticides include pyrethroids sprayed by truck, and organophosphates sprayed by airplane. Larvicides are used to treat water sources containing mosquito larvae with water-soluble insecticide formulations. More chemical classes of larvicides exist compared to adulticides, the most common of which include bacterial metabolites, synthetic growth hormones, and surface contact oils.

Larvicides are often used as a first line of defense in mosquito control (Marcombe et al. 2014), serving as a preemptive strategy that can destroy potential vectors of disease before they are capable of spreading. Larvicides may be attractive to mosquito control districts due to the ability to rotate between different products, which can assist in curbing the development of resistance (Georghiou 1994). Many larvicides are provided in slow release formulations, which allows for residual insecticidal effects. However, there are issues with larviciding programs. The lipophilicity and poor water solubility of many insecticides hinders their ability to stay suspended in the organically dense aquatic substrates that larval *Culex* mosquitoes live within (Cleveland et al. 2002). One of the most popular larvicides, *Bacillus thuringiensis* subspecies *israelensis*, readily binds to organic matter and settles to the bottom of the water column within days, reducing the likelihood of mosquito larvae ingesting the toxin and receiving a lethal dose (Lacey 2007). In addition to these efficacy issues, spinosad (Jones and Ottea 2013) and temephos (Marina et al. 2014) have been found to cause significant non-target mortality in aquatic Coleoptera, Odonata,

and Hemiptera at mosquito larvicide label application rates. Examination of these larviciding issues are essential for modern day mosquito control districts.

Mosquito control was first established in our study area of East Baton Rouge Parish in 1979 (EBRMARC 2016). In the present study, we hypothesized that the water quality of septic water that *Cx. quinquefasciatus* prefer to oviposit within had an effect on larvicide efficacy. This hypothesis was tested by replicating septic water parameters in the lab, followed by exposing a reference strain of *Cx. quinquefasciatus* to an experimentally derived diagnostic concentration. The results of this study may help inform local mosquito control districts as to potential strengths and shortcomings of their larvicide treatments in field water conditions.

3.2 Materials and Methods

3.2.1 Chemicals

Acetone (pesticide grade) was purchased from Amresco (Solon, OH). Technical grade spinosad (65.56% spinosyn A, 31.37% spinosyn D; 96.9%) was purchased from Sigma-Aldrich (St. Louis, MO). Formulations of *Bacillus sphaericus* 2362 (VectoLex® WDG, 51.2%) and temephos (ABATE® 4-E, (O,O,O,O-Tetramethyl O,O-sulfanediylbis(1,4-phenylene) diphosphorothioate), 44.6%) were donated by East Baton Rouge Mosquito Abatement and Rodent Control (Baton Rouge, LA). Lactalbumin and brewer's yeast were donated by the Livingston Parish Mosquito Abatement District (Denham Springs, LA). Composted manure (Gardenese® Compost & Manure), dry dog food (Purina® Beneful), sucrose (Great Value™ Pure Cane Sugar), magnesium sulfate (PL Developments® Epsom Salt), acetic acid (Great Value™ Distilled White Vinegar), and sodium bicarbonate (Espoma® Garden Lime) were purchased locally.

3.2.2 Insects

East Baton Rouge Mosquito Abatement and Rodent Control provided a susceptible, reference strain of *Cx. quinquefasciatus* (henceforth Sebring) to the Louisiana State University medical entomology lab in January of 2015 as egg rafts. This colony has since been maintained in the medical entomology insectary within the Life Sciences building on the Louisiana State University campus. The Sebring strain originates from Sebring, FL and was originally collected and maintained by the local USDA Agricultural Research Station (Stancil 2000).

Egg rafts of *Cx. quinquefasciatus* were transferred to white plastic trays (25 x 35 cm) filled with 1.5 liters of diH₂O that had been exposed to open air for ≥24 h. Trays were incubated at 27°C (±2) and a 14h light:10h dark cycle. A mixture of 75 mg lactalbumin and 75 mg brewer's yeast was sprinkled on to the water surface every weekday for food. Following pupation, pupae were removed individually by pipetting, and transferred to shallow glass cups that were then placed in collapsible cages (31cm³) inside of the medical entomology insectary, set to 27°C (±2) and 14h light:10h dark.

Adult mosquitoes were provided 10% sugar solution *ad libitum* in 150 ml Erlenmeyer flasks with cotton wicks. Sugar water was refreshed twice weekly, and wicks changed weekly. Cages containing adults were draped in damp cotton cloth and shrouded partially by plastic bags in order to increase humidity. A Hobo® (Onset Computer Corporation, Bourne MA) sensor placed near adult cages monitored potential deviations in temperature and light cycle. Sugar was removed from cages one day prior to blood feeding, after which defibrinated chicken-blood (Rockland™ Immunochemicals, Limerick, PA) was provided for ≥2 hours, using an artificial feeder (Hemotek® Ltd, England) with stretched

Parafilm M® (Bemis Company, Oshkosh, WI) as a membrane. Following blood feeding, a shallow black cup containing aged diH₂O was inserted into the cage for oviposition. Egg rafts were later removed from the cups to white plastic trays.

3.2.3 Water Quality Sampling Sites

Water quality parameters were measured at eleven sites in northern East Baton Rouge Parish (Figure 8) in an attempt to recreate septic water conditions in sterile water for use in a BSL1 laboratory. Measurements were also taken from lab tap water as a means of comparison. Sites were chosen with the assistance of East Baton Rouge Mosquito

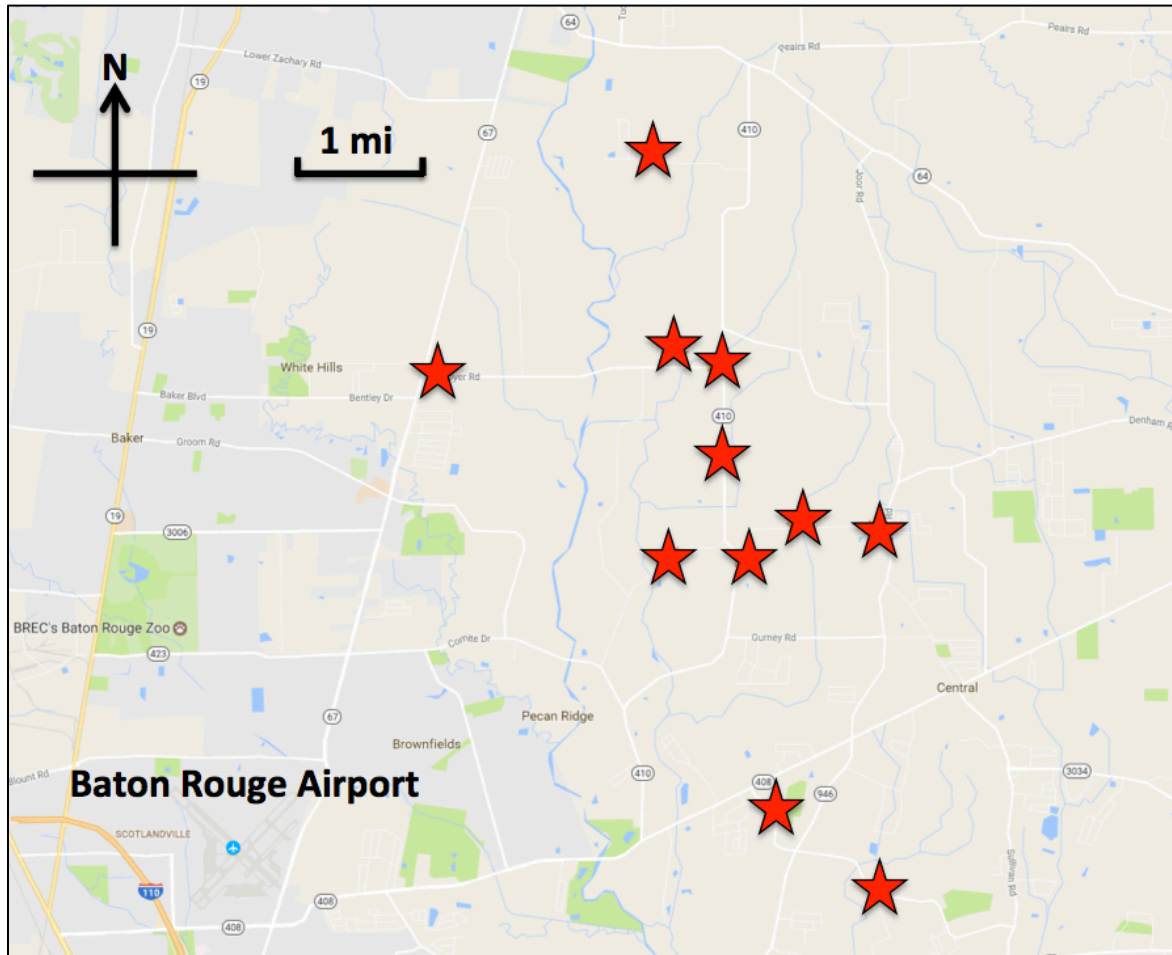


Fig. 8. Map of water quality sampling sites in East Baton Rouge Parish. Rendered with Google Maps.

Abatement and Rodent Control, and were located in areas that received consistently high numbers of larvicide treatments for *Cx. quinquefasciatus*. All sites were located in drainage ditches in the front yards of residential houses. Septic drainage pipes from residential households were observed emptying into all sites (Figure 9). Measurements were taken from sites between 7 March and 18 March 2016.



Fig. 9. Picture of a water quality sampling site: drainage ditch with white septic outlet pipe. Lovett Rd, East Baton Rouge Parish, LA.

Water quality parameters were measured using a handheld pH/water quality meter (Hach® Lange H-170). Measurements taken include: temperature (°C), pH, reduction potential (mV), total dissolved solids (ppm), conductivity (µs/cm), and salinity (ppt). Turbidity was measured by eye on a scale from 0 – 5: zero when the bottom was clearly visible, and five when completely obfuscated by sediments (Table 5). Replication of water quality parameters in sterile lab conditions consisted of trial and error from mixing various volumes of diH₂O and: composted manure (Gardenese® Compost & Manure), dry dog food

(Purina® Beneful), sucrose (Great Value™ Pure Cane Sugar), magnesium sulfate (PL Developments® Epsom Salt), acetic acid (Great Value™ Distilled White Vinegar), and sodium bicarbonate (Espoma® Garden Lime). Susceptibility bioassays were performed in the septic water analog on the Sebring strain using the experimentally derived LC₉₉ of each larvicide as a diagnostic concentration.

Table 5. Water quality measurements from sites in East Baton Rouge Parish.

Site	Temp (C)	pH	Reduction potential (mV)	Total dissolved solids (ppm)	Conductivity (µs/cm)	Salinity (ppt)	Turbidity
Core 1	19	6.9	13	230	470	0.23	3
Core 2	20	7.3	-10	110	220	0.11	5
Lovett 1	21	7.3	-7.5	160	310	0.15	2
Lovett 2	21	7.5	-16	170	340	0.16	0
Sonny 1	23	7.4	-4.5	190	390	0.18	3
Sonny 2	21	6.9	18	72	150	0.07	3
High	22	7.5	-16	100	210	0.10	4
BW	21	7.8	-39	130	270	0.13	5
Dyer	23	7.6	-20	130	260	0.12	2
Carey	21	7.5	-18	110	220	0.11	0
Blanka	21	7.1	9.0	170	340	0.17	1
Site	21	7.4	-8.4	140	290	0.14	3
Average							
Lab Tap	26	7.4	-0.40	0.97	2.0	0	0
Average							

3.2.4 Biological Assays

Stock solutions (10,000 ppb) of *Bacillus sphaericus* and temephos were diluted in ddH₂O and stored in amber bottles at room temperature. A stock solution (100,000 ppb) of spinosad was diluted in ACS grade acetone and stored in an amber bottle at room temperature. Stock solutions were serially diluted with distilled water immediately prior to bioassay.

Initial susceptibilities of the Sebring colony were measured in Pyrex® glass petri dishes (No. 3140-100; 10x5 cm). Comparisons were made between glass and paper

containers by performing bioassays again within 200 ml paper cups (Karat® 6oz paper food containers). Paper containers were used for the remainder of this study. Comparisons between concentration response lines performed in glass and paper containers are available in the Appendix.

Approximately 20 late-3rd and/or early-4th instar *Cx. quinquefasciatus* were removed from rearing trays with mesh nets, and placed into containers holding 100 ml of diH₂O. Following addition of larvae, larvicides were aliquoted into each cup, giving desired final concentrations. Cups containing 100 ml of diH₂O were used as controls. Plastic wrap was placed over cups to prevent desiccation, and cups were then incubated at 27°C and a 14h light:10h dark cycle. Mortality readings were taken after 24 hours for spinosad and temephos, and 48 hours for *Bacillus sphaericus*. Larvae were considered dead if unresponsive upon prodding with a sharp pencil tip.

Insecticide susceptibility of larvae from the Sebring colony was reasoned using triplicate bioassays and at least seven different concentrations. Final concentrations of insecticides in the assays ranged from 0.01 – 10 ppb for *Bacillus sphaericus*, 1 – 5,000 ppb for spinosad, and 0.01 – 10 ppb for temephos. Susceptibilities were measured over four to five different determination dates using freshly prepared concentrations of insecticides.

Concentrations of insecticide that killed approximately 99% of reference Sebring mosquitoes (LC₉₉) was determined by probit analysis (Hoskins and Craig 1962) for each larvicide, and were used as diagnostic concentrations to measure the effect of water quality on Sebring mortality. For these assays, treatments used 20 larvae (in triplicate) in 100 ml of an artificial septic water analog, and were treated with the LC₉₉ of *Bacillus sphaericus*

(9 ppb), spinosad (1,700 ppb), and temephos (4.5 ppb). Triplicate bioassays were performed over three determination dates, with fresh stocks of insecticide mixed for each date. As a means of comparison, the mortality response of the Sebring colony to the reference LC₉₉ was also measured in diH₂O (in triplicate) over three separate determination dates. Cups containing approximately 20 late-3rd and/or early-4th instar *Cx. quinquefasciatus* and either 100 ml of diH₂O or 100 ml of septic water analog were used as controls. Mortality was recorded over at least three separate determination dates with newly mixed concentrations of insecticides.

3.2.5 Statistical Analysis

All statistical analyses were performed using MiniTab® ver 17 (Minitab 2016). Mortality data from the Sebring colony were subjected to probit analysis, calculating slope, LC₅₀ and LC₉₉ values. Student's T-tests were used to compare mortality data following exposure of the reference Sebring strain to diagnostic concentrations of insecticides in a septic water analog and diH₂O.

3.3 Results

3.3.1 Susceptibility to *Bacillus sphaericus*, spinosad, and temephos

The reference Sebring colony was subjected to varied concentrations of larvicides for probit analysis, producing baseline LC₅₀ and LC₉₉ values for *Bacillus sphaericus*, spinosad, and temephos (Figure 10). The Sebring colony was most susceptible to *Bacillus sphaericus* (LC₅₀=0.21ppb), followed by temephos (LC₅₀=0.55ppb), and was least susceptible to spinosad (LC₅₀=50ppb). LC₉₉ values obtained in response to treatment with *Bacillus sphaericus* (LC₉₉=9ppb), spinosad (LC₉₉=1,700ppb), and temephos (LC₉₉=4.5ppb) were used as diagnostic concentrations to measure differences in *Cx. quinquefasciatus*

mortality in diH₂O and a septic water analog. Comparisons between concentration response lines performed in glass and paper containers are available in the Appendix.

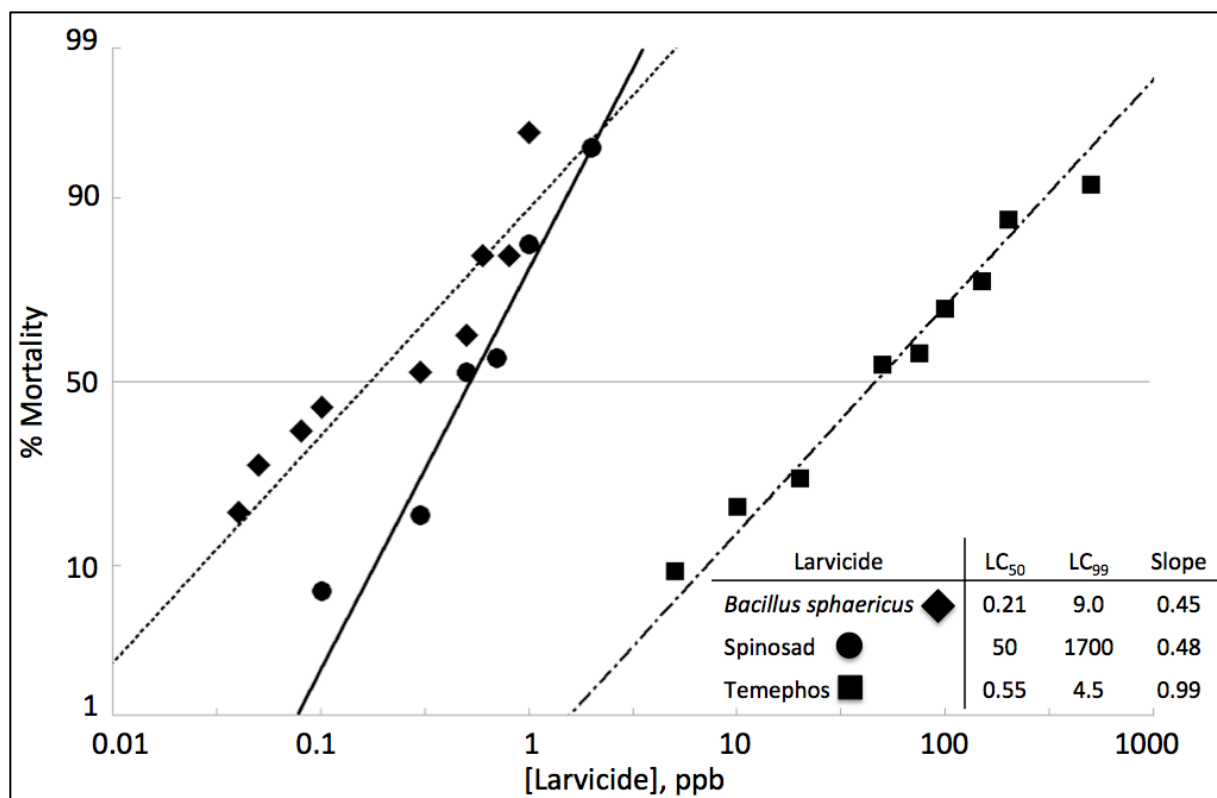


Fig. 10. Susceptibility of the reference Sebring colony to *Bacillus sphaericus*, spinosad, or temephos. Data were collected from at least 8 concentrations of larvicide, with at least 20 larvae treated in triplicate over 3-5 separate determination dates. LC₅₀ and LC₉₉ were determined using probit analysis.

3.3.2 Water Quality

The recipe that was found to most closely approximate average septic water conditions in the field contained 10 liters of lab tap water and 1 liter of composted manure (Table 6). Total dissolved solids and salt content were elevated in the septic analog (220 ppm and 0.30 ppt, respectively) compared to average site water (140 ppm and 0.14 ppt, respectively). However, compared to lab tap water and other recipes, this particular recipe remained the closest analog of field septic water conditions.

Table 6. Comparison of water quality measurements from sites in East Baton Rouge Parish, an artificially created septic water analog, and lab water.

Site	pH	Reduction potential (mV)	Total dissolved solids (ppm)	Conductivity ($\mu\text{s}/\text{cm}$)	Salinity (ppt)	Turbidity
Site Average	7.4	-8.4	140	290	0.14	3
Septic Analog Average	7.3	-14	220	455	0.30	2
Lab Tap Average	7.4	-0.40	0.97	2.0	0	0

Mortality in response to the diagnostic Sebring LC₉₉ of *Bacillus sphaericus*, spinosad, and temephos was not significantly impacted by water type (Table 7). Compared to clean lab water, mean mortality was lower, and standard deviation higher, in septic water that had been treated with *Bacillus sphaericus* and temephos. However, none of these differences were statistically significant.

Table 7. Susceptibility of the Sebring reference strain in septic or clean water.

Sebring Mortality (\pm SD)								
<i>Bacillus sphaericus</i>			Spinosad			Temephos		
Septic	Clean	P-value	Septic	Clean	P-value	Septic	Clean	P-value
93 (\pm 6)	96 (\pm 5)	0.62	99 (\pm 1)	99 (\pm 1)	0.96	91 (\pm 10)	99 (\pm 2)	0.49

Mortality was measured in response to a diagnostic concentration (Sebring reference strain LC₉₉) of *Bacillus sphaericus*, spinosad, or temephos. Means were obtained by treating 20 larvae in triplicate with the diagnostic LC₉₉ in septic or clean H₂O, over three determination dates. Student's T-tests ($\alpha=0.05$) were used to compare the effect of water on mortality.

3.4 Discussion

Sampling 11 field septic ditches over the course of two weeks allowed for measurement of mean water quality parameters. On average, septic water pH was found to be similar to tap water; other parameters (reduction potential, total dissolved solids, conductivity, salinity, turbidity) were markedly different. Replication of septic water

parameters with sterile lab components allowed for testing without concerns for sanitary or disease issues associated with human septic water, such as hepatitis A, which would require utilization of BSL-2 facilities (CDC 2009). However, with the development of a septic water analog, this trial lost immeasurable value in no longer being a true field efficacy trial.

Larval *Cx. quinquefasciatus* that had been treated with the reference, diagnostic LC₉₉ for *Bacillus sphaericus*, spinosad, or temephos were not significantly affected by water quality. This was expected for *Bacillus sphaericus*, as its spores are persistent in sediment rich waters compared to other larvicides (Yousten et al. 1992). Similarly, formulations of temephos have been used extensively in sediment rich water sources (Lacorte et al. 1996) as a result of their persistence in the water column. A study using formulations of spinosad in field microcosms and mesocosms found that spinosad maintained efficacy in different water sources, but that residual activity decreased with lower concentrations (Jiang and Mulla 2009). In combination with the results from this study, these data suggest that even technical grade, unformulated spinosad may be able to function at concentrations similar to the reference Sebring LC₉₉ in semi-field conditions.

Future studies would benefit from actual field data in septic conditions, although risk of infection when dealing with human excrement may be a cause for concern (Jewitt 2011). Additionally, mortality data from biological assays using diagnostic concentrations of larvicide were likely insufficient in measuring the impact of water quality parameters on larvicide efficacy. Similar studies evaluated the effect of different concentrations of larvicide in semi-field scenarios (Jiang and Mulla 2009), as opposed to the single diagnostic concentration used in this study. While insect mortality can be an indicator of insecticide

distribution within a medium, changing each water quality parameter (i.e. total dissolved solids) in isolation may have better informed the effect of septic water on larvicide efficacy.

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SUMMARY AND CONCLUSION

Mosquito vectored parasites and pathogens cause morbidity and death around the world, but mosquito control has made an impact. Malaria, which killed 1.2 million people in Africa in 2010 alone, was endemic to the United States as recently as 70 years ago. As was discovered with applications of DDT, which was used heavily in the elimination of vectors of malaria from the United States, extensive insecticide use inevitably results in the development of resistance. Mosquito control districts need to maintain the susceptibility of their mosquito populations, lest they be faced with an uncontrollable population of insect vectors.

East Baton Rouge Mosquito Abatement and Rodent Control treats *Culex quinquefasciatus*, the primary vector of West Nile virus in the southern United States, with larvicides year-round. One of their most prominent larvicides, *Bacillus sphaericus*, was sprayed at one experimental site at least 16-times throughout the summer of 2016 alone. Maintaining larvicide susceptibility is important, especially for vectors of disease causing agents. The goal of this study was to evaluate larvicide efficacy in semi-field scenarios, and to examine the susceptibility of mosquito larvae in East Baton Rouge Parish to three commonly used larvicides: *Bacillus sphaericus*, spinosad, and temephos.

Culex quinquefasciatus from East Baton Rouge Parish were found to be susceptible to both *Bacillus sphaericus* and spinosad. Furthermore, this susceptibility did not change over the course of the 2016 mosquito season. Considering the amount of *Bacillus sphaericus* used historically, as well as in 2016 alone, these data indicate that mosquito control has so far avoided a detectable frequency of resistance. Spinosad was used infrequently to control mosquito larvae in Baton Rouge in 2016. Many mosquito control

districts are only now beginning to incorporate it into their programs, following its labeling as a mosquito larvicide in 2007. Finding no change in susceptibility to spinosad in the field was unsurprising, yet positive with regards to future use of spinosad in Louisiana.

Culex quinquefasciatus from one site in East Baton Rouge Parish, and all three sites in Livingston Parish, were found to have high frequencies of resistance to the organophosphate temephos. This discovery was surprising considering temephos is not used as a mosquito control product in Louisiana. Upon further examination of the Baton Rouge population, 5-fold temephos resistance was detected in the spring, which increased to 10-fold over the course of the 2016 mosquito season. We hypothesized that lowered susceptibility to temephos may have arisen due to cross-resistance from other pesticide applications. Organophosphates were not sprayed for mosquito control at the resistant site in Baton Rouge, but the pyrethroid resmethrin was used extensively. Cross-resistance has been observed in *Cx. quinquefasciatus* between both pyrethroids and organophosphates as a result of increased esterase activity. Enzymatic measurements from the resistant mosquito population in Baton Rouge indicated an elevated level of esterase activity, providing evidence into how susceptibility may have decreased in the wild.

Considering *Bacillus sphaericus* and spinosad remain effective against local *Cx. quinquefasciatus*, and since temephos isn't used in Louisiana, local mosquito control seems to be properly curbing the development of larvicide resistance. Additionally, all larvicides from this study appeared effective in the septic environment in which *Culex* females are known to oviposit. The discovery of temephos resistance without temephos treatment raises some concern regarding the selective pressures exerted on local mosquitoes, and may be an exciting avenue for further investigation.

APPENDIX: GLASS/PAPER CUP COMPARISONS

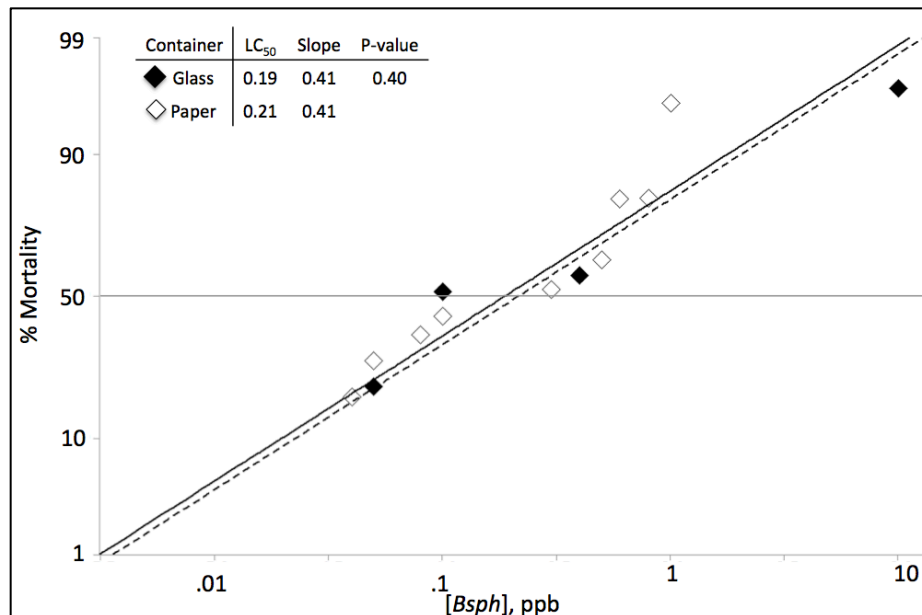


Fig. 11. Susceptibility of the reference Sebring colony in response to varied concentrations of *Bacillus sphaericus* in glass and paper cups. At least 20 mosquitoes were treated in triplicate per determination. LC₅₀ and LC₉₉ were determined using probit analysis. Probit Z-test ($\alpha=0.05$) was used to examine whether lines of regression were identical. P-value <0.05 indicates lines are significantly different from one another.

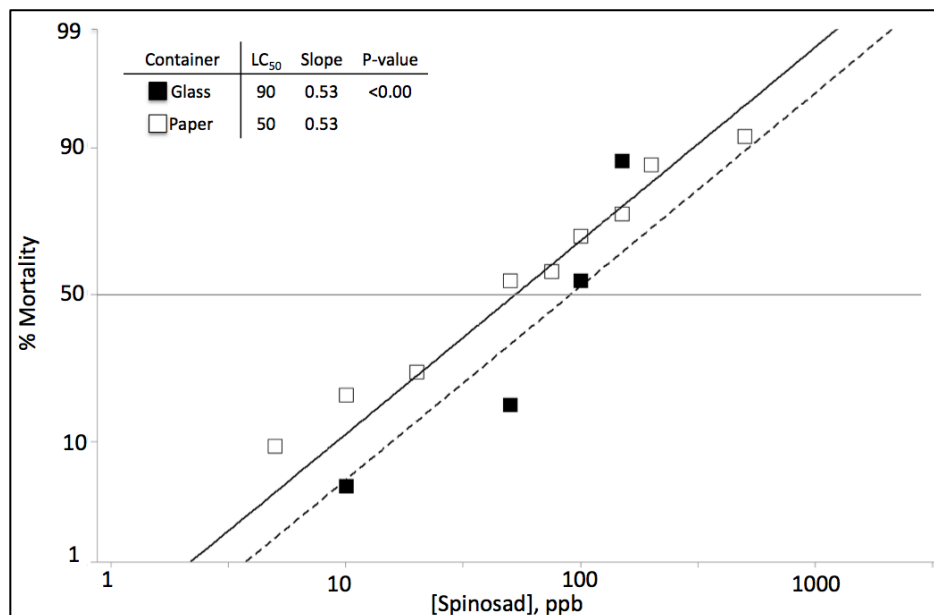


Fig. 12. Susceptibility of the reference Sebring colony in response to varied concentrations of spinosad in glass and paper cups. At least 20 mosquitoes were treated in triplicate per determination. LC₅₀ and LC₉₉ were determined using probit analysis. Probit Z-test ($\alpha=0.05$) was used to examine whether lines of regression were identical. P-value <0.05 indicates lines are significantly different from one another.

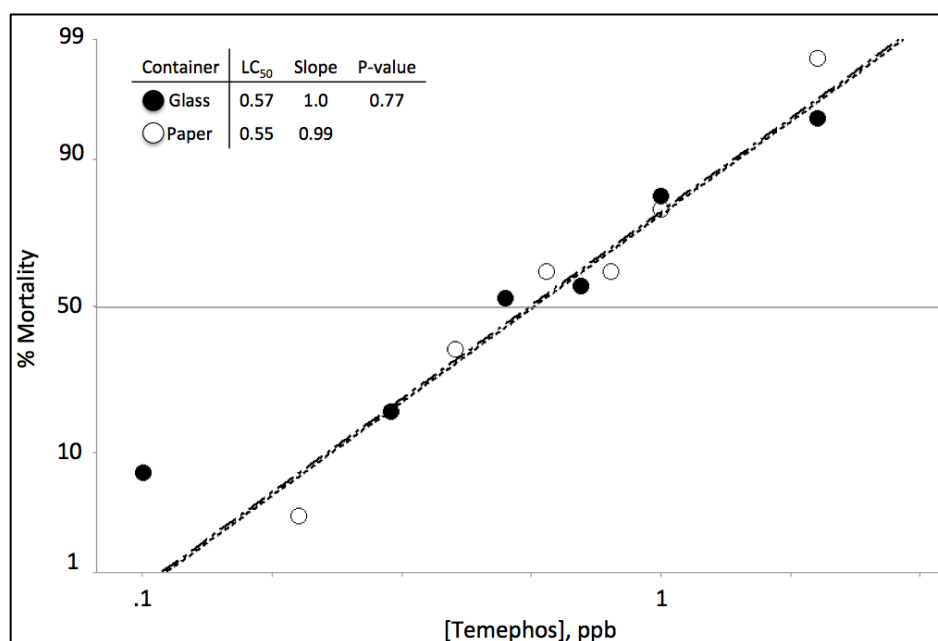


Fig. 13. Susceptibility of the reference Sebring colony in response to varied concentrations of temephos in glass and paper cups. At least 20 mosquitoes were treated in triplicate per determination. LC₅₀ and LC₉₉ were determined using probit analysis. Probit Z-test ($\alpha=0.05$) was used to examine whether lines of regression were identical. P-value <0.05 indicates lines are significantly different from one another.

VITA

Nicholas DeLisi was born in 1990 in Salt Lake City, Utah. After graduating with a bachelor's of science in biology from the University of Utah, Nick spent two years working for the Salt Lake City Mosquito Abatement District. While there, he gained a deep appreciation for entomology, and a hatred for mosquitoes. After two years of working in Salt Lake City, Nick applied for graduate school under the advising of Dr. Kristen Healy at Louisiana State University. While in Louisiana, Nick honed his mosquito hunting skills, began writing about himself in the third person, and made plenty of new friends. He plans to graduate with a Master's degree in entomology in May of 2017. In June of 2017, Nick will begin working for the St. Tammany Parish Mosquito Abatement District as an entomologist.