IMPACTS OF AN ORGANOPHOSPHATE AND A PYRETHROID ON INSECTICIDE RESISTANCE IN *CULEX QUINQUEFASCIATUS* (DIPTERA: CULICIDAE)

by

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ABSTRACT

Environmental security is a large part of public health. While an abundance of research has been done to assess insecticide resistance in adult mosquitoes, there is a general lack of information about the potential effects of insecticide exposure on immature (i.e., larval) mosquitoes. Chemical control and reducing the number of larval habitats are two of the most successful ways to keep mosquito populations under control. In this project, we investigated whether exposure to sub-lethal doses of insecticides at the larval stage impacted insecticide resistance in mosquitoes at the adult stage using a colony of insecticide-susceptible *Culex quinquefasciatus* mosquitoes obtained from the Centers for Disease Control and Prevention (CDC). Approximately 10 larvae/well were placed into a six well plate. Ten six well plates were used for each group (total of 30 plates) and filled with tap water that had been inoculated with insecticide. Two different types of insecticides were used: Fyfanon® (organophosphate; active ingredient [AI] malathion) and Biomist® (pyrethroid; AI permethrin). A control group not exposed to any insecticides was also included. Once the larvae had developed into adults, a CDC bottle assay was completed to determine the extent to which adult mosquitoes were resistant or susceptible to the insecticides. Following the CDC bottle bioassay protocol, the number of dead mosquitoes was recorded at several pre-determined time points for up to two hours. This was done for all three groups. After the bottle bioassay was completed, a line graph was made for visual interpretation of data. For the mosquito population tested here, mosquitoes exposed to insecticides as larvae were more likely to be susceptible to the insecticides as adults.

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Impacts of an Organophosphate and a Pyrethroid on Insecticide Resistance in *Culex quinquefasciatus* (Diptera: Culicidae)

Introduction

As a public health student, diseases and vector control are of special interest to me. Environmental security is a huge part of public health and that includes keeping individuals and the setting they live in as safe as possible. In order to control the mosquito population, representative populations of each prevalent species must be tested in order to determine the extent to which insecticide resistance exists we well as to what types of insecticides they may be resistant. While an abundance of research has been done on adult mosquitoes and their insecticide resistance, there seems to be a lack of completed research on mosquito larvae and potential effects of exposing them to insecticides while still in this stage. Chemical control and reducing the number of larval habitats are two of the most successful ways to keep mosquito populations under control. In this project, a laboratory experiment was done to investigate the extent to which exposure to sub-lethal doses of insecticides at the larval stage impacts insecticide resistance in mosquitoes at the adult stage.

Background

Living in Eastern North Carolina, mosquitos are no stranger to our area. The *Culex quinquefasciatus* mosquito, better known as the southern house mosquito, is one of the more common to our state and can transmit a multitude of pathogens that cause diseases that impact human and veterinary health (e.g., West Nile virus, Eastern equine encephalitis virus). *Culex quinquefasciatus* typically ranges from 3.96 to 4.25 mm in length and is brown in color. Female *Culex* mosquitoes lay egg rafts in standing water which can be in places such as old tires, bird baths, retention ponds, and ditches (Hamdan, Sofian-Azirun, Nazni & Lee, 2005). Because the

larvae of *Cx. quinquefasciatus* are found in standing water (e.g., retention ponds), there is an interest in potential run-off of insecticides into these areas which could impact resistance/susceptibility profiles. My experiment used six well plates to simulate the standing water environment in which mosquito live determined the extent to which larval exposure to insecticides impacted adult resistance/susceptibility profiles.

Purpose

To begin this study, environmental stressors on mosquitoes were researched, specifically in *Culex quinquefasciatus*, to determine the extent to which stressors may impact insecticide resistance. These stressors can vary depending on the location so my project focused on Greenville, North Carolina. There are four broad classes of insecticides used all over the world in treating mosquitoes: they are carbamates, pyrethroids, organochlorines, and organophosphates (National Pesticide Information Center, 2016). In this experiment, a pyrethroid (permethrin) and an organophosphate (malathion) were tested.

Through this experiment, my goal was to investigate how mosquitoes become resistant to insecticides and whether exposure as larvae to the specific substance impacted the resistance they had as an adult mosquito. I expected to see results indicating that mosquitoes that have been exposed to insecticides as immature life stages (i.e., larvae) would be more resistant to the insecticides as adults, compared to larvae not exposed to insecticides. This is due to the fact that insecticides are commonly used in the Pitt County area and mosquitoes may develop resistance to insecticide AIs (Richards, Balanay, Fields & Vandock, 2017). The goal was not to kill the larval mosquitoes with the insecticide, but to administer a sub-lethal dose to them in order to assess whether it affects resistance profiles.

Methodology

This experiment used a colony of insecticide-susceptible *Culex quinquefasciatus* mosquitoes obtained from the CDC and maintained following standard protocols. These mosquitoes were blood fed one time per month and the larvae they produced were used for the actual experimentation along with the adult mosquitoes. In order to do this successfully, some preliminary calculations and experiments were completed to determine the correct insecticide dosages needed for exposure of the mosquito larvae. A serial ten-fold dilution was done on the first batch of mosquitoes in order to find correct dosage for both Fyfanon® and Biomist®.

Dosage Experiment 1

The experiment began in August 2018 when six six-well plates were prepared to allow a plate for Fyfanon® 10^{-2}, Fyfanon® 10^{-3}, Fyfanon® 10^{-4}, Biomist® 10^{-2}, Biomist® 10^{-3}, and Biomist® 10^{-4}. Six 1st instar larvae were put into the first well of each plate and five 1st instar larvae were placed into the second and third wells of each plate. The plates had been filled with tap water that had been inoculated with insecticide. For both Fyfanon and Biomist, a tenfold serial dilution was performed as follows:

10^{-2} plate: 70 µL insecticide + 7 mL water

10^{-3} plate: 7 µL insecticide + 7 mL water

10^{-4} plate: 0.7 µL insecticide + 7 mL water

The 70 µL volume was added using a pipette that ranged from 20-200 µL while the 7 and 0.7 µL volumes were added using a pipette that ranged from 0-20 µL. The Fyfanon® stock was at 400 µL/mL whereas Biomist® stock was at 8 µL/mL. Each plate was placed into a 28° incubator. For the following three days, the six-well plates were inspected and a count was taken/recorded of how many larvae were dead in each well. See Table 1 below for a three-day count of the

larvae. Due to the unexpected high mortality rates, a second experiment was done to further adjust insecticide dosage.

	Day 0			Day 1			Day 2			Day 3		
	Plates											
	1	2	3	1	2	3	1	2	3	1	2	3
Number Alive				Number Dead (% mortality)								
Fyfanon®												
10^{-2}	6	5	5	6 (100)	5 (100)	5 (100)	6 (100)	5 (100)	5 (100)	6 (100)	5 (100)	5 (100)
10^{-3}	6	5	5	6 (100)	5 (100)	5 (100)	6 (100)	5 (100)	5 (100)	6 (100)	5 (100)	5 (100)
10^{-4}	6	5	5	0 (0)	5 (100)	5 (100)	0 (0)	5 (100)	5 (100)	0 (0)	5 (100)	5 (100)
Biomist®												
10^{-2}	6	5	5	6 (100)	5 (100)	5 (100)	6 (100)	5 (100)	5 (100)	6 (100)	5 (100)	5 (100)
10^{-3}	6	5	5	3 (50)	0 (0)	5 (100)	4 (66)	1 (20)	5 (100)	4 (66)	1 (20)	5 (100)
10^{-4}	6	5	5	0 (0)	5 (100)	5 (100)	0 (0)	5 (100)	5 (100)	0 (0)	5 (100)	5 (100)

Table 1

Dosage Experiment 2

The second dosage experiment was done in September 2018. Three six-well plates were prepared. A pipette was used to dispense 7 mL of 10^{-4} Fyfanon® stock solution and 7 mL of 10^{-4} Biomist® stock solution into wells of respective plates. The third plate served as a control plate, with seven millimeters of tap water added to each well. Each plate was placed into a 28° incubator for approximately 45 minutes in order to bring the solutions up to temperature. Approximately ten second instar *Culex quinquefasciatus* mosquito larvae were placed into each well of the three six-well plates. The larvae were fed using a 2:1 ratio of yeast:liver powder and the plates were placed into the incubator. For the next three days, the larvae were counted daily to determine the mortality rate. See Table 2 for results.

	# dead (% mortality)			
	Day 0 Number Alive	Day 1	Day 2	Day 3
Biomist® 10^{-4}				
Well 1	10	0 (0)	0 (0)	0 (0)
Well 2	10	3 (30)	3 (30)	4 (40)
Well 3	10	1 (10)	1 (10)	2 (20)
Well 4	10	0 (0)	0 (0)	0 (0)

Well 5	10	0 (0)	1 (10)	1 (10)
Well 6	10	0 (0)	1 (10)	2 (20)
Fyfanon® 10^{-4}				
Well 1	10	7 (70)	9 (90)	9 (90)
Well 2	13	10 (77)	13 (100)	13 (100)
Well 3	11	8 (73)	10 (91)	10 (91)
Well 4	15	8 (53)	14 (93)	14 (93)
Well 5	16	6 (38)	14 (88)	15 (94)
Well 6	18	18 (100)	18 (100)	18 (100)
Control				
Well 1	11	8 (73)	8 (73)	8 (73)
Well 2	12	8 (67)	9 (75)	9 (75)
Well 3	11	3 (27)	5 (45)	5 (45)
Well 4	13	4 (31)	6 (46)	6 (46)
Well 5	12	1 (8)	2 (16)	2 (16)
Well 6	10	4 (40)	5 (50)	5 (50)

Table 2

A low percentage of the Biomist® 10^{-4} larvae died while a high percentage of the Fyfanon® 10^{-4} larvae died. The control group also had a higher mortality rate than normal. For these reasons, a third trial experiment with a lower dose of Fyfanon® and a higher dose of Biomist® was completed.

Dosage Experiment 3

In the final dosage experiment, four six-well plates were prepared. A pipette was used to put 7 mL of 10^{-5} Fyfanon® stock solution into each well of this plate. A new pipette was used to put 7 mL of 10^{-6} Fyfanon stock solution into each well of the second plate. A new pipette was used to put 7 mL of Biomist® 10^{-4} stock solution into each well of the third plate. The fourth plate served as a control plate, with 7 mL of tap water in each well. Each plate was placed into a 28° incubator for approximately 45 minutes in order to bring the solutions up to temperature. Approximately ten second instar *Culex quinquefasciatus* larvae were placed into each well of the four six-well plates. The larvae were fed with a liver powder (as described previously) and the

wells were placed into the incubator. For the next five days, the larvae were counted daily to determine the mortality rate. See Table 3 below for results.

	# dead (% mortality)						
	Day 0 Number Alive	Day 1	Day 2	Day 3	Day 4	Day 5	Mean Mortality (%)
Biomist 10^{-4}							
Well 1	9	0 (0)	0 (0)	1 (11)	1 (11)	1 (11)	6.6
Well 2	13	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 3	12	0 (0)	0 (0)	1 (8)	1 (8)	1 (8)	4.8
Well 4	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 5	9	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 6	12	0 (0)	0 (0)	2 (16)	2 (16)	2 (16)	9.6
Fyfanon 10^{-5}							
Well 1	17	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 2	15	0 (0)	0 (0)	1 (6)	1 (6)	1 (6)	3.6
Well 3	9	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)	2.2
Well 4	11	0 (0)	0 (0)	2 (18)	2 (18)	2 (18)	10.8
Well 5	13	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 6	12	10 (83)	10 (83)	10 (83)	10 (83)	10 (83)	83
Fyfanon 10^{-6}							
Well 1	19	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 2	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 3	12	0 (0)	0 (0)	1 (8)	1 (8)	1 (8)	4.8
Well 4	13	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 5	11	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 6	14	0 (0)	0 (0)	1 (7)	1 (7)	1 (7)	4.2
Control							
Well 1	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 2	9	0 (0)	0 (0)	1 (11)	1 (11)	2(22)	8.8
Well 3	11	0 (0)	0 (0)	2 (18)	2 (18)	3(27)	12.6
Well 4	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Well 5	10	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	2
Well 6	9	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0

Table 3

Overall, the mortality rates were much lower than previous trials completed. Well 6 of the Fyfanon 10^{-5} group was accidentally overfed when the larvae were put into the wells which accounts for the much higher mortality rate observed in this well.

After confirming the correct dosages, the main experiment began in October 2018. Thirty six-well plates were prepared. A pipette was used to put 7 mL of 10^{-5} Fyfanon® stock solution into each well of 10 plates. A new pipette was used to put 7 mL of 10^{-4} Biomist® stock solution into each well of 10 plates. The final 10 plates served as controls, with 7 mL of tap water in each well. Each plate was placed into a 28° incubator for approximately 45 minutes in order to bring the solutions up to temperature. Approximately ten *Culex quinquefasciatus* mosquito larvae, second instar, were placed into each well of the 30 six-well plates. The larvae were fed with a liver powder and the wells were placed into the incubator. For the next four days, the larvae were counted to determine the mortality rate. See Table 4 below for results.

			# dead (% mortality)				
		Number Alive Day 0	Day 1	Day 2	Day 3	Day 4	Mean Mortality (%)
Biomist® 10^{-4}							
Plate 1	A	9	0	0	0	0	0
	B	10	0	0	0	0	0
	C	12	0	0	0	0	0
	D	9	0	0	0	0	0
	E	11	0	0	0	0	0
	F	10	0	0	0	0	0
Plate 2	A	10	0	0	0	0	0
	B	13	0	0	0	0	0
	C	12	0	0	1(8.3)	0	2.075
	D	11	0	0	0	0	0
	E	13	0	0	0	0	0
	F	12	0	0	0	0	0
Plate 3	A	12	0	0	0	0	0
	B	14	0	0	0	0	0
	C	9	0	0	0	0	0
	D	13	0	0	0	0	0

	E	14	0	0	0	0	0
	F	10	0	0	0	0	0
Plate 4	A	10	0	0	0	0	0
	B	9	0	0	0	0	0
	C	9	0	0	0	0	0
	D	10	0	0	0	0	0
	E	10	1(10)	1(10)	2(20)	2(20)	15
	F	10	0	0	0	0	0
Plate 5	A	14	0	0	0	0	0
	B	11	0	0	0	0	0
	C	10	0	0	0	0	0
	D	8	0	0	0	0	0
	E	8	0	0	0	0	0
	F	12	0	0	0	0	0
Plate 6	A	13	0	0	0	0	0
	B	10	0	0	0	0	0
	C	10	0	0	0	0	0
	D	10	0	0	0	0	0
	E	10	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 7	A	12	1(8.3)	1(8.3)	1(8.3)	1(8.3)	8.3
	B	11	0	0	0	0	0
	C	10	0	0	0	0	0
	D	10	0	0	0	0	0
	E	11	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 8	A	10	0	0	0	0	0
	B	10	0	0	0	0	0
	C	10	0	0	0	0	0
	D	9	0	0	0	0	0
	E	9	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 9	A	12	0	0	0	0	0
	B	11	0	0	0	0	0
	C	15	1(6.6)	1(6.6)	1(6.6)	1(6.6)	6.6
	D	12	0	0	0	0	0
	E	12	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 10	A	12	0	0	0	0	0
	B	12	0	0	0	0	0
	C	14	0	0	0	0	0
	D	12	0	0	0	0	0
	E	13	0	0	0	0	0
	F	11	0	0	0	0	0
Fyfanon® 10^{-5}							

Plate 1	A	10	0	0	0	0	0
	B	10	0	0	0	0	0
	C	8	0	0	0	0	0
	D	11	0	0	0	0	0
	E	10	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 2	A	9	0	0	0	0	0
	B	9	0	0	0	0	0
	C	10	0	0	0	0	0
	D	-	-	-	-	-	-
	E	7	0	0	0	0	0
	F	10	0	0	0	0	0
Plate 3	A	9	1(11)	1(11)	3(33)	3(33)	22
	B	9	0	0	0	0	0
	C	8	0	0	0	0	0
	D	9	0	0	0	0	0
	E	10	0	0	0	0	0
	F	9	0	1(11)	2(22)	2(22)	13.75
Plate 4	A	10	0	0	0	0	0
	B	9	0	0	0	0	0
	C	10	0	0	0	0	0
	D	10	0	0	0	0	0
	E	10	0	0	0	0	0
	F	9	0	0	0	0	0
Plate 5	A	11	0	0	0	0	0
	B	10	0	0	0	0	0
	C	10	0	0	0	0	0
	D	11	0	0	0	0	0
	E	9	0	0	0	0	0
	F	9	0	0	0	0	0
Plate 6	A	12	0	0	0	0	0
	B	9	0	0	0	0	0
	C	11	0	0	0	0	0
	D	8	0	0	0	0	0
	E	10	0	0	0	0	0
	F	13	0	0	0	0	0
Plate 7	A	10	0	0	0	0	0
	B	12	0	0	0	0	0
	C	17	0	0	0	0	0
	D	11	0	0	0	0	0
	E	10	0	0	0	0	0
	F	13	0	0	0	0	0
Plate 8	A	9	0	0	0	0	0
	B	11	0	0	0	0	0
	C	10	0	0	0	0	0

	D	10	0	0	0	0	0
	E	12	0	0	0	0	0
	F	14	0	0	0	0	0
Plate 9	A	11	0	0	0	0	0
	B	12	0	0	0	0	0
	C	12	0	0	0	0	0
	D	13	0	0	0	0	0
	E	17	2(11.7)	2(11.7)	2(11.7)	3(17.6)	13.175
	F	17	0	0	0	0	0
Plate 10	A	12	0	0	0	0	0
	B	11	0	0	0	0	0
	C	15	0	0	0	0	0
	D	19	0	0	0	0	0
	E	16	0	0	0	0	0
	F	12	0	0	0	0	0
Control							
Plate 1	A	12	0	0	0	1(8.3)	2.075
	B	10	0	0	0	0	0
	C	10	0	0	0	0	0
	D	10	0	0	0	0	0
	E	10	0	0	0	0	0
	F	13	0	0	0	0	0
Plate 2	A	10	0	0	0	0	0
	B	10	0	0	0	0	0
	C	10	0	0	0	0	0
	D	12	0	0	0	0	0
	E	10	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 3	A	10	0	0	0	0	0
	B	9	0	0	0	0	0
	C	10	0	0	0	0	0
	D	10	0	0	0	0	0
	E	9	0	0	0	0	0
	F	11	0	0	0	0	0
Plate 4	A	10	0	0	0	0	0
	B	12	0	0	0	0	0
	C	10	0	0	2(20%)	2(20%)	10
	D	10	0	0	0	0	0
	E	10	0	0	0	0	0
	F	10	0	0	0	0	0
Plate 5	A	10	0	0	0	0	0
	B	12	0	0	0	0	0
	C	11	0	0	0	0	0
	D	12	0	0	0	0	0
	E	10	0	0	0	0	0

	F	10	0	0	0	0	0
Plate 6	A	10	0	0	0	0	0
	B	10	0	0	0	0	0
	C	10	0	0	0	0	0
	D	10	0	0	0	0	0
	E	9	0	0	0	0	0
	F	10	0	0	0	0	0
Plate 7	A	12	0	0	0	0	0
	B	11	0	0	0	0	0
	C	11	0	0	0	0	0
	D	10	0	0	0	0	0
	E	16	0	0	0	0	0
	F	12	0	0	0	0	0
Plate 8	A	13	0	0	0	0	0
	B	13	0	0	0	0	0
	C	12	0	0	0	0	0
	D	10	0	0	0	0	0
	E	10	0	0	0	0	0
	F	12	0	0	0	0	0
Plate 9	A	12	0	0	0	0	0
	B	16	0	0	0	0	0
	C	13	0	0	0	0	0
	D	15	0	0	0	0	0
	E	11	0	0	0	0	0
	F	18	0	0	0	0	0
Plate 10	A	9	0	0	0	0	0
	B	10	0	0	0	0	0
	C	11	0	0	0	0	0
	D	10	1(10)	1(10)	1(10)	1(10)	10
	E	10	0	0	0	0	0
	F	10	0	0	0	0	0

Table 4

During the last week of October 2018, pupae began to be transferred from six well plates to cages. There are 3 cages for the control group, 3 for the Biomist® group, and 3 for the Fyfanon® group. In each cage is a small plastic cup with a small amount of tap water in which I transferred the pupae to. Once all pupae were transferred to their respective cages, I placed a sugar ball containing a 20% sucrose solution on the top of each one. Pupae were transferred

each day until November 7th, 2018. The larvae were fed every third day with liver powder and the sugar balls on the cages were replaced every third day.

On November 7th, 2018 bottles were prepared for the bottle bioassay. Twelve 500 mL glass Wheaton bottles total were used with four being coated in acetone, four in Biomist®, and four in Fyfanon®. The bottle bioassay began at exactly 8:00 am on November 8th, 2018. Approximately ten female mosquitos were placed into each bottle. Two bottles were used to test mosquitoes pre-exposed to Biomist® in Biomist® (8 µg/mL) coated bottles, two bottles for Fyfanon® exposed mosquitoes in Fyfanon® (400 µg/mL) coated bottles, two bottles for unexposed mosquitoes in control (acetone) bottles, two bottles for control mosquitoes in Fyfanon® (400 µg/mL) coated bottles, two bottles for control mosquitoes in Biomist® (8 µg/mL) coated bottles, one bottle Fyfanon® exposed mosquitoes in an acetone coated bottle, and one bottle Biomist® exposed mosquitoes in an acetone coated bottle. Timers were started for each set of bottles and a count was taken every 15 minutes to determine how many mosquitos had died at each interval for up to a two hour period following CDC bottle bioassay protocols.

Results and Discussion

Below is Figure 1 that shows mortality curves from the CDC bottle bioassay experiment. My original hypothesis was that adult mosquitoes exposed to insecticide as larvae would live longer when exposed as adults compared to adults that were not pre-exposed to insecticides as larvae. I thought the larval pre-exposure would increase the adult resistance due to upregulation of detoxification enzymes, providing a protective effect on the mosquitos as adults. The experiment proved this to be quite the opposite, however. Pre-exposure as larvae to the insecticides increased adult susceptibility to that insecticide when compared to control mosquitoes who had no pre-exposure to insecticide. It is possible that pre-exposure to

insecticides as larvae weakened the detoxification response of the same adult mosquitoes, hence resulting in higher susceptibility to insecticides.

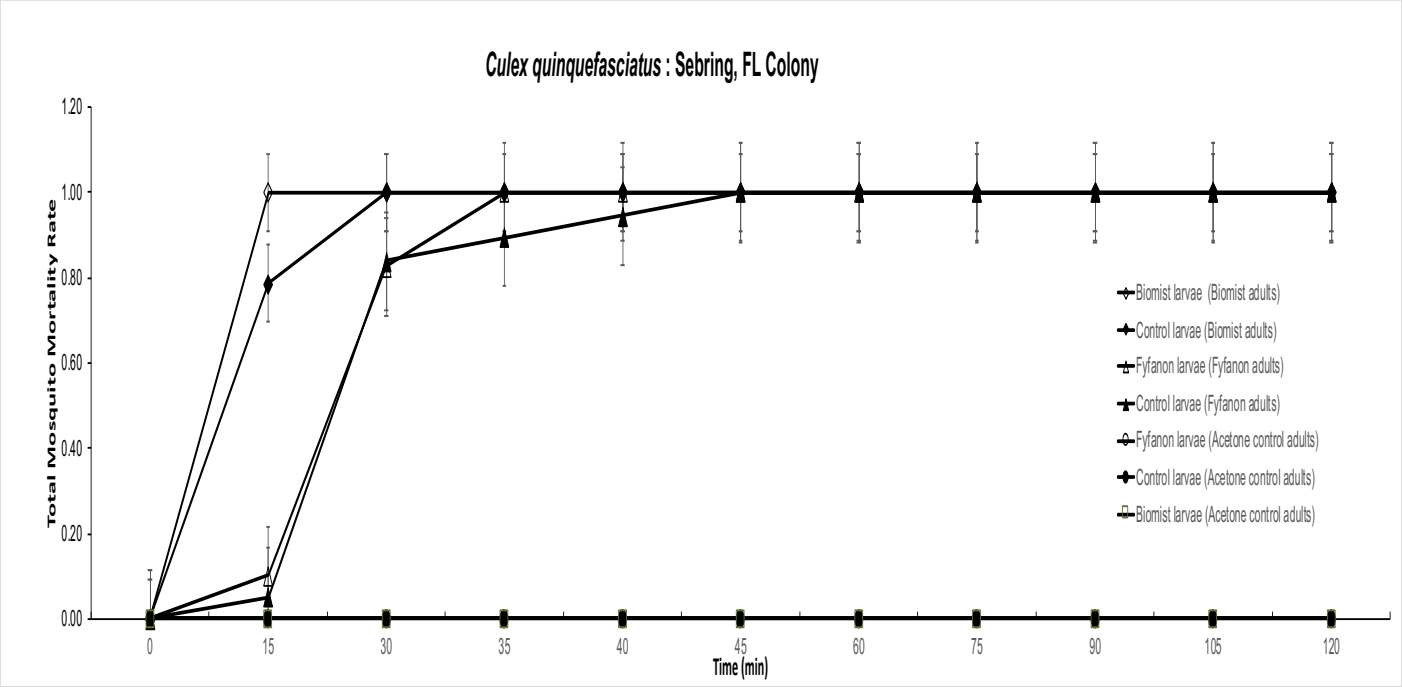

Figure 1

Conclusion

It is important to note that although all efforts were made to simulate the natural environment of the *Culex quinquefasciatus* mosquito, it was ultimately completed in a laboratory which could affect results. Although the results did not agree with the hypothesis in this experiment, the information gained is still useful for research purposes as well as for vector control specialists when attempting to figure out the best way to limit mosquitoes on farms and in residential areas.

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