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The Effect of Intermediate Doses of Roundup® on the Larval Development, Survival, Oviposition and Fitness of Culex quinquefasciatus

Abdoulaye Aziz Zerbo

THE EFFECT OF INTERMEDIATE DOSES OF ROUNDUP® ON THE LARVAL DEVELOPMENT, SURVIVAL, OVIPOSITION AND FITNESS OF *CULEX QUINQUEFASCIATUS*

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Public Health

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by
Abdoulaye Aziz Zerbo
August 2024

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ABSTRACT

The southern house mosquito, *Culex quinquefasciatus*, is a vector to many pathogens in Southern California, including West Nile Virus and St. Louis Encephalitis Virus. Immature stages of mosquitoes may be exposed to Roundup®, a frequently used herbicide worldwide, due to surface runoffs or overspraying and subsequent drifting. Previous studies have shown some negative impacts of sublethal doses of Roundup® during the developmental stage of *Culex quinquefasciatus*, such as physiologic changes and prolonged larval stage or the high mortality of larvae due to exposure to the high doses of glyphosate. This study investigated the effect of intermediate doses of Roundup® (10 mg/l and 100 mg/l) on *Culex quinquefasciatus* when immature stages are exposed to this herbicide. Experiments were conducted in the Environmental Chambers at CSU San Bernardino at 27 ± 2°C, RH 70 ± 5%, and 12:12 hour Photoperiod.

Results indicated prolongation of the larval period and delays in pupation upon exposure to the above concentrations of Roundup®. The results also indicated that as the dose of glyphosate increased, the yield of adult emergence decreased. Oviposition experiments have shown fewer egg rafts oviposited as Roundup® concentration increased. Another interesting observation was a

significant increase in the adult wingspan when the exposure dose at the larval stage was increased from 10 mg/l to 100 mg/l.

ACKNOWLEDGEMENTS

I want to express my heartfelt gratitude to my family and friends for their unwavering support throughout my academic journey. Their encouragement and belief in my abilities have continuously strengthened and motivated me. My profound gratitude also goes to Dr. Mahmood Nikbakhzadeh and my Co-researcher, Mayra Espinoza, who guided me tremendously during this journey.

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CHAPTER ONE
INTRODUCTION

Problem Statement

Some of the most devastating infectious diseases, including dengue fever, lymphatic filariasis, and malaria, are transmitted by mosquitoes (Foster et al., 2019). Mosquitoes do not provide parental care to offspring, and therefore, natural selection should favor the ability of gravid females to select aquatic habitats that will maximize offspring fitness and egg hatching (Yee et al., 2020; Kibuthu et al., 2016). Chemical pollutants can potentially affect this process by modifying the quality and attractiveness of aquatic habitats. Vector biologists have examined the impact of pollutants such as glyphosate on mosquitos' behavior, ecology, and pathogen transmission ability (Kibuthu et al., 2016).

The southern house mosquito, *Culex quinquefasciatus*, is widespread in Southern California and is a vector to many pathogens, including West Nile Virus and St. Louis Encephalitis Virus (Bhattacharya & Basu, 2016). Mosquitoes have four different life stages, including egg, larva, pupae, and adult. Immature mosquito stages, including larvae, eggs, and pupae, are susceptible to water contaminants (Nikbakhtzadeh & Fuentes, 2022). *Culex quinquefasciatus* deposits her eggs in stagnant water, which makes it highly vulnerable to environmental pollutants. Further, their breeding sites are usually shielded by some vegetation (Manimegalai & Sukanya, 2014). When mosquitoes are exposed to glyphosate, there may be changes in the life characteristics of mosquitoes, changes in their behavior, larval reproduction, or survival rate (Bara et al., 2014). In addition, exposure to glyphosate can lead to developing pesticide resistance (Bataillard et al., 2020). Such changes can eventually change the mosquito vectorial capacity (Nikbakhtzadeh & Fuentes, 2022).

Purpose of Study

This study examines the effect of 10,000 µg/l (10 mg/l) and 100.000 µg/l (100 mg/l) glyphosate solutions on *Culex quinquefasciatus.*

Research Questions

1. What effect will a 10,000 µg/l or 100.000 µg/l of glyphosate solution have on the larval development of *Culex quinquefasciatus*?
2. What effect will a 10,000 µg/l or 100,000 µg/l glyphosate solution have on adult emergence of *Culex quinquefasciatus*?
3. What will be the effect of such glyphosate concentrations on the oviposition and hatching rate of *Culex quinquefasciatus*?
4. Will a concentration of 10,000 µg/l or 100,000 µg/l significantly affect the fitness of *Culex quinquefasciatus*?

Significance to Public Health

Mosquitoes are hematophagous insects that are important in public health because they can transmit various pathogens to the human population. Hundreds of infectious pathogens have been found in mosquitoes; however, less than 100 pathogens cause diseases in humans (Nebbak et al., 2022). In the

United States, vector-borne diseases have increased over the past decade; from 2004 to 2016, nine new mosquito-borne diseases were discovered (Rosenberg et al., 2018).

Culex quinquefasciatus is a geographically abundant, widespread, and epidemiologically significant vector for various groups of pathogens, including protozoa, filarial worms, and arboviruses. This species is the primary vector of *Plasmodium relictum* (malaria parasite in birds), *Wuchereria bancrofti,* and lymphatic filariasis. *Culex quinquefasciatus* is widely distributed in the subtropics and tropics and represents a significant threat to human and animal health (Harvey-Samuel et al., 2021). Although the number of people infected with the St. Louis and West Nile viruses is less than the number of people infected with some other mosquito-borne diseases, the economic costs of vector control, disease surveillance, and medical expenses to local governments are high (Barber et al., 2010). *Culex quinquefasciatus* can also transmit pathogens such as Rift Valley fever, fowl pox, West Nile encephalitis, fowl malaria, and canine heartworm disease (LaPointe, 2012) to pets and livestock, causing loss of productivity and animal death.

Since its introduction into the United States in 1999, the West Nile virus has resulted in more than 2,000 deaths (Cockerill et al., 2017). Even though there is an effective vaccine against the Japanese encephalitis virus, an estimated 68,000 cases occur annually in Asia (Moore, 2021). The presence of mosquitoes in human habitats and their capacity to transmit deadly pathogens to human communities justifies prioritizing these studies as significant public health concerns (Dahmana & Mediannikov, 2020).

CHAPTER TWO
LITERATURE REVIEW

Culex quinquefasciatus

Culex quinquefasciatus is a mosquito species living in watersheds in tropical and subtropical urban regions. *Culex quinquefasciatus* females seek ephemeral, organically rich, or permanent stagnant water to deposit their eggs. *Culex quinquefasciatus* is a semi-domestic species found near human dwellings because of the presence of sewers, septic ditches, and various sources of standing water (Medeiros-Sousa et al., 2015). *Culex quinquefasciatus,* which is widespread throughout the southern United States (both urban and rural areas) (López-Solis et al., 2023), is predominantly a bird feeder. However, they can also feed on other vertebrates, including humans (Hannon et al., 2019).

Roundup®

Roundup® is an herbicide with the active ingredient Glyphosate that inhibits the synthesis of amino acids in the shikimic pathway of plants, leading to excess shikimic acid production, which in turn causes plants' death (Vivancos et al., 2011). Glyphosate is the most widespread agricultural herbicide in the world, where approximately 150 thousand tons are applied annually in the United States alone (Atwood & Paisley-Jones, 2017). Due to the excessive use of glyphosate, it can easily leave the target site and enter stagnant water in different concentrations (Bataillard et al., 2020). Over the years, glyphosate has been used in large quantities, which led to widespread environmental contamination.

The offsite presence of glyphosate threatens different ecosystems and non-target organisms (Zhan et al., 2018). Large-scale leakage of glyphosate into terrestrial and aquatic environments has occurred because of excessive and improper application practices (Hanke et al., 2010). Glyphosate is technically non-toxic to birds and mammals. However, invertebrates, including insects, are very sensitive to it (Morris et al., 2016).

Culex quinquefasciatus and Glyphosate Research

A laboratory study of the effect of glyphosate on *Culex quinquefasciatus* showed that a glyphosate concentration of 1 gram/liter (g/l) of glyphosate was lethal to larvae or larvae were unable to molt to pupae or never emerged as adult mosquitoes (Nikbakhtzadeh & Fuentes, 2022). *Culex quinquefasciatus* larvae that were exposed to a concentration of 0.5 g/l glyphosate experienced the same effects, while the larval stage was extended and pupation was significantly delayed (Nikbakhtzadeh & Fuentes, 2022). *Culex quinquefasciatus* that were oviposited in 0.5 g/l glyphosate deposited the same number of egg rafts as in the control (water), while a significantly lower number of eggs hatched to first instar larvae in glyphosate (Nikbakhtzadeh & Fuentes, 2022). If gravid females laid eggs in 1 (g/l) of glyphosate compared to the control, the difference between the two groups would be statistically significant, and only a small number of eggs laid in glyphosate were ever developed into larvae in the glyphosate solution (Nikbakhtzadeh & Fuentes, 2022). If glyphosate solutions and water are offered as oviposition mediums to *Culex quinquefasciatus* in a multiple-choice assay, gravid females first select water, then the lower, and finally, the higher concentrations (Nikbakhtzadeh & Fuentes, 2022).

According to a study by Kibuthu et al. (2016), *Culex quinquefasciatus* eggs are usually smaller when exposed to glyphosate. Similarly, egg raft occurrence in the glyphosate solution was significantly lower compared to water (Kibuthu et al., 2016). Multivariate analysis of variance for both sexes of mosquitoes showed a significant impact of agrochemicals on the development time to adulthood, as well as on longevity and wing length (Kibuthu et al., 2016). *Culex quinquefasciatus* females had a longer development time in the control group than in the glyphosate-treated group. Females treated with glyphosate were about the same size as females from the control group (Kibuthu et al., 2016). Glyphosate had no statistically significant effects on the longevity of *Culex quinquefasciatus* females (Kibuthu et al., 2016). The development time of male *Culex quinquefasciatus* treated with glyphosate was shorter compared to the control group (Kibuthu et al., 2016).

Another study showed that male *Culex quinquefasciatus* exposed to glyphosate four days after eclosion indicated a significant reduction in sperm production, while there was no effect on sperm production on day one, seven, or 14 days after eclosion in mosquitoes in the laboratory (Judd, 2018). This study additionally showed that glyphosate did not significantly affect sperm production in wild populations of mosquitoes (Judd, 2018).

Glyphosate and Other Mosquito Species

Exposure of mosquito larvae to agricultural chemicals is a widespread phenomenon. However, the impact of chemicals used in agriculture on mosquito larvae is still poorly understood, especially how the behavior and ecology of mosquitoes can be altered after exposure to such chemicals (Bara et al., 2014). Previous research has already shown that exposure of *Culex pipiens* mosquitoes to field-realistic doses of glyphosate did not affect larval survival rate, female fecundity, and the size of adult mosquitoes. However, exposure to glyphosate reduced the development time and the probability that the mosquito would become infected with the malaria parasite (Bataillard et al., 2020). In *Anopheles arabiensis*, glyphosate exposure reduced the pupation time in an insecticide-resistant strain (Oliver & Brooke, 2018). Treatment with glyphosate increased the longevity of mosquitoes that were resistant to insecticides. In adult mosquitoes, larvae exposed to glyphosate act as an enhancer of insecticide tolerance (Oliver & Brooke, 2018). Glyphosate exposure had a contrasting effect on detoxification enzyme activities. Glyphosate increased catalase activity and decreased glutathione S-transferase activity in mosquitoes (Oliver & Brooke, 2018).

A study showed a positive synergistic effect on *Aedes aegypti* larval mortality when treated with glyphosate and *Bacillus sphaericus* simultaneously (Bernal & Dussán, 2020). However, further molecular analyses are needed to understand the mechanism of this synergistic effect (Bernal & Dussán, 2020). After exposure to glyphosate, tolerance to permethrin and imidacloprid increased in mosquito larvae (Riaz et al., 2009). Larval tolerance was slightly increased to propoxur after exposure to glyphosate. Cytochrome p450 monooxygenase activities were moderately induced in mosquito larvae exposed to glyphosate and imidacloprid (Riaz et al., 2009).

Glutathione S-transferase activity in mosquito larvae was strongly induced after exposure to propoxur and moderately induced after larval exposure to glyphosate (Riaz et al., 2009). Esterase activities in larvae were slightly induced after exposure to glyphosate and significantly induced after exposure to propoxur (Riaz et al., 2009). Research on the effect of glyphosate field-realistic doses on *Aedes aegypti* showed a dose-dependent harmful effect of glyphosate (Baglan et al., 2018).

In another study, the authors reared 150 first-instar *Aedes aegypti* and *Aedes albopictus* larvae in 1.6 L live oak leaf infusion with or without 5 mg/l glyphosate or atrazine (Bara et al., 2014). Here, containers were observed daily to monitor sex ratio, emergence rate, female body size, and emergence time of females and males. The occurrence rates of *Aedes aegypti* after atrazine treatment were significantly higher than the control and glyphosate treatments. In contrast, the occurrence rate of *Aedes albopictus* in the atrazine-treated group was significantly higher than in the glyphosate treatment. In both mosquito species, the authors observed a skewed sex ratio with a male bias in the glyphosate-treated and control groups but not in the atrazine-treated group.

In short, it is essential to investigate the effects of glyphosate on mosquitoes, as agrochemicals may impact their biology and behavior significantly and thus affect vectorial competence and capacity. Although many studies have been conducted on the effects of glyphosate on mosquitoes, only a few studies have examined the effect of glyphosate on *Culex quinquefasciatus*. Depending on the concentration, glyphosate may induce different effects on mosquito larvae. Therefore, further research is required to determine the whole range of glyphosate effects on mosquitoes' behavior and life cycle.

CHAPTER THREE
METHODS

Study Design

This research was conducted at the Mosquito Rearing Facility of California State University, San Bernardino, directed by Dr. Nikbakhtzadeh. Several critical procedures were implemented to optimize the setting for the research activities. These involved intense chamber cleansing, sterilization, and the regulation of the heater and humidifier to obtain the appropriate levels of heat and humidity. The lab's preparatory phase for mosquito propagation involved extensive disinfection of all areas. All plastic containers were regularly cleaned with hot water using an abrasive sponge (Scotch-Brite, Tonawanda, NY) and then left to dry. For nutritional support, adult mosquitoes were provided with a 10% sucrose solution (w/v), and the female specimens also received defibrinated bovine blood meals (Na citrate blood; LAMPIRE Biological Laboratories, Pipersville, PA) to reproduce. The ambient conditions, temperature, humidity, and photoperiod were continuously monitored and fine-tuned to cater to the needs of the mosquito population and to preserve an environment favorable to their development and survival. This was overseen daily, and adjustments were made whenever necessary. The initial eggs of the *Culex quinquefasciatus* species were obtained from the Northwest Mosquito and Vector Control District (NWMVCD) in Corona, CA.

Data Source and Collection

To mimic the natural environmental conditions of the mosquitoes, the experimental conditions were carefully controlled for temperature, moisture, and light exposure. The research occurred in a climate-controlled environmental chamber where the temperature was consistently regulated to 27 ± 2°C using a heater (Holmes HCH 4062B, Boca Raton, FL). The humidity level was sustained at 70 ± 5% through humidifiers (Levoit LV600S, Anaheim, CA, and Elechomes SH8820, Newark, CA). Using a programmed timer, the light cycle was set to alternate following a 12-hour light to a 12-hour dark regimen.

After the eggs hatched, groups of 120 newly emerged first instar larvae were collected (Fig 1A) and placed into plastic containers (29.3 × 19.7 × 6 cm) filled with 750 ml of aged tap water (Fig 1B). The feeding protocol of larvae followed the dietary guidelines by Gary and Foster (2001) with some modifications.

Figure 1. A: Counting larvae to prepare the larval containers   B: Larval containers, each containing 120 larvae.

Within seven days, the larvae developed to the fourth instar, after which they molted into pupae. These pupae were carefully collected and transferred to the BioQuip field cages (40 x 40 x 40 cm, BioQuip Products, Inc., Compton, CA) in plastic pupation containers (4 5/8 x 3 inches). Two days later, the pupae molted into adult mosquitoes (Fig 2). These adults were supplied with cotton rolls soaked in tap water and 10% (w/v) sucrose solution.

Figure 2: Adult cages with 400 specimens each. Vials of water and 10% sucrose solution were provided to each cage.

Four to five days after adult emergence, females were fed with defibrinated bovine blood (Na-citrate bovine blood, Lampire Biological Laboratories, Pipersville, PA) on two consecutive days to have a more successful blood feeding.

Experiment Set Up

To assess the effects of glyphosate, two concentrations of 10 and 100 mg/l were prepared. Twenty-first instar larvae were put into single-use plastic containers with a volume of 400 ml and a diameter measuring 10.8 cm, and they were filled with 200 ml of the two concentrations of the glyphosate solution at the respective concentrations. In contrast, the control containers received only aged tap water to serve as a standard for comparison, as shown in Figure 3.

Figure 3. Larval experiment: The containers with the blue tab contained the glyphosate solutions (treatment), whereas the ones with green tabs had just water (Control)

Each experimental and control setup had ten replicates (n=10). To prepare the 10 mg/l and 100 mg/l glyphosate solutions, 23.2 and 232 µg/l of Glyphosate Super Concentrate (Roundup® Weed and Grass Killer, 50.2% glyphosate isopropyl amine salt, Monsanto, Marysville, OH) were thoroughly mixed with 1000 ml of tap water respectively. Both the treatment and control set of larvae received a food regimen of 0.02 g of TetraMin® (Tetra GMBH, Herrenteich, D-49324, Melle, Germany) fish food, administered daily during the first four days. After the fourth day, until pupation, they received a food regimen of 0.06 g. Daily data recording was employed to track the larvae through their developmental milestones, including mortality, pupation, and adult emergence, ensuring a complete oversight of our experimental mosquito growth. These records permitted a detailed analysis of the developmental trends among the glyphosate-treated and control larvae by calculating the molting ratios, thus offering a numerical perspective on larval survival, molting procedure, and development within each set of replicates. The meticulous data recording and analytical process were pivotal in uncovering the influence of the test factors on the mosquitoes' development and survival rates.

For blood meal preparation, 40℃ water was poured into 250 ml PYREX glass bottles. Then, a small quantity of blood was dispensed onto the concave bottom of the bottle, which was immediately covered by a very thin layer of Parafilm® (Millipore Sigma, St. Louis, MO). This allows mosquitoes to pierce through this artificial membrane when the bottle is placed upside down atop the mosquito enclosure (Fig 4). By using this artificial membrane feeding system, we did not need to use live animals, which eased the whole process and exempted us from obtaining an IRB certification.

Figure 4. Preparation of blood feeding apparatus. A: n-citrate bovine blood, B: blood apparatus preparation, C: Blood is offered to each adult cage

Data Analysis

This thesis entailed a detailed statistical examination to investigate the larval survival and development rates, egg oviposition preferences of gravid females, and morphometric analyses of adult specimens. The Kaplan-Meier and Wilcoxon tests were utilized to create the larval survival curves. Kolmogorov-Smirnov normality test was used to determine if any data sets had a normal distribution. If so, Analysis of Variance (ANOVA), followed by the Waller-Duncan posthoc test and Student t-test, were consequently used. If data did not indicate a normal distribution, then the Mann-Whitney U test was used to compare experimental groups versus control. Analyses were performed using SPSS software (version 29.00, Chicago, Illinois), and Microsoft Excel (version 18.2110.13110.0, Redmond, WA) were used to illustrate graphics.

CHAPTER FOUR

RESULTS

This chapter describes the effect of two concentrations of Roundup® on the survival, development, oviposition, and fitness of *Culex quinquefasciatus*.

Larval Development

Larval survival rate after exposure to glyphosate solutions was assessed and compared to the control groups.

For the 10,000 µg/l glyphosate solution, the average number of live larvae (mean= 5.6) was non-significant compared to the number of live larvae represented by the control group (mean = 5.5) during the entire experiment (Fig 5A). When it comes to the 100,000 µg/l glyphosate solution, the average number of live larvae (mean = 5.59) was also non-significant compared to the number of live larvae represented by the control group (mean = 5.45) (Fig 5B)

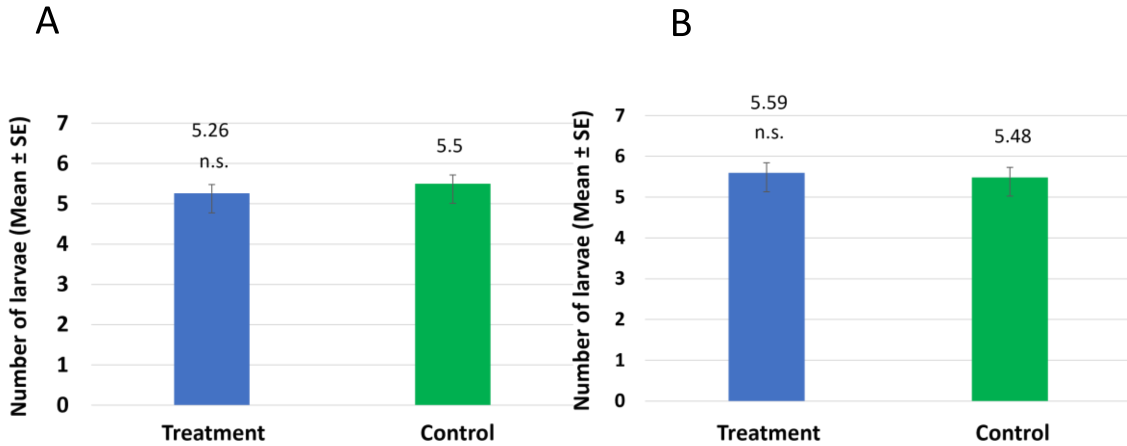

Figure 5. Mean ± SE of live larvae of treatment versus control *Culex quinquefasciatus* during the whole experiment (n=10). (A): 10 mg/l glyphosate solution (treatment) vs water (control). Kolmogorov-Smirnov Test, Z= 0.671, P= 0.759; Paired sample t-test (df= 9, t= 0.414, P= 0.688). (B): 100 mg/l glyphosate solution (treatment) vs water (control). Kolmogorov-Smirnov Test, Z= 0.894, P= 0.4; Paired sample t-test (df= 9, t= 0.222, 2-tailed P= 0.829).n.s. on a bar indicates a non-significant difference.

Molting Ratio

This test evaluated the impact of the two glyphosate solutions on the molting ratio of *Culex quinquefasciatus* larvae and pupae. The approach used the Kaplan-Meier survival estimator and further analysis through the Wilcoxon test.

For the 10,000 µg/l glyphosate versus control, the development of treated larvae was slower than the control until day 7, when the pace started to be about the same (Fig 6A). Pupation started a little earlier for the treatment, around day 6, while the control group started around day 7 (Fig 6A). Both treatment and control reached a steady pace for their development around day 10.

At 100,000 µg/l concentration, the development of treated larvae was slower than the control until day 8, when the pace started to be about the same (Fig 6B). Pupation started somewhat earlier for the treatment, around day 6, while for the control group, it started around day 8 (Fig 6B).

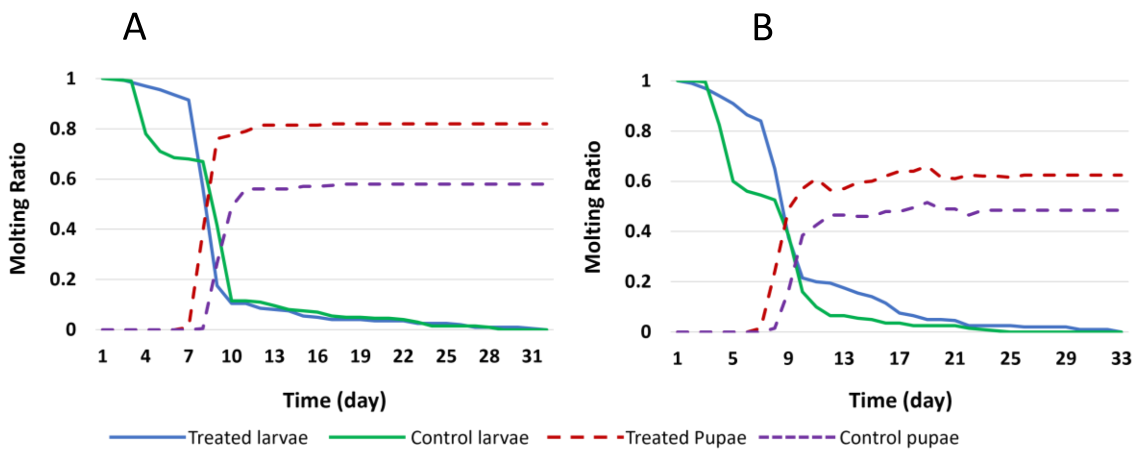

Figure 6. Molting ratio of *Culex quinquefasciatus* larval instars exposed to (treatment) versus water (control). Kaplan-Meier survival estimator, followed by Wilcoxon test. (A): 10 mg/l glyphosate solution (treatment) vs water (control); (B): 100 mg/l glyphosate solution (treatment) vs water (control).

Average Counts of Live Larvae and Pupae

These results are represented graphically. The blue and green graphs represent the number of live larvae, and the red and purple graphs represent the number of live pupae.

The analysis of the 10,000 µg/l glyphosate solution revealed no significant difference between the daily count of live larvae (mean = 5.4, df=30, t-value= 0.762) compared to the control group (mean = 5.14, P>0.05). As far as pupation, the study indicated a P-value of 0.003 resulting from the Kolmogorov-Smirnov test suggesting a significant difference between the treatment (mean=974) and the control (mean =6.17), but the Mann-Whitney U test (Z=1.983, P< 0.051) indicates that these differences are not significant. (Fig 7A).

In the case of 100,000 µg/l, the paired sample t-test analysis of the average daily count of larvae (mean=5.59, df=25, t-value=4.169) was significantly higher at a 99% confidence level compared to the control (mean=5.48 P<001). On the other hand, pupation, the average daily count of pupae in the treatment (mean=8.07, Z=2.557), showed a significant difference at a 95% confidence level compared to the control (mean=5.86, P<0.05), as shown in Figure 7B.

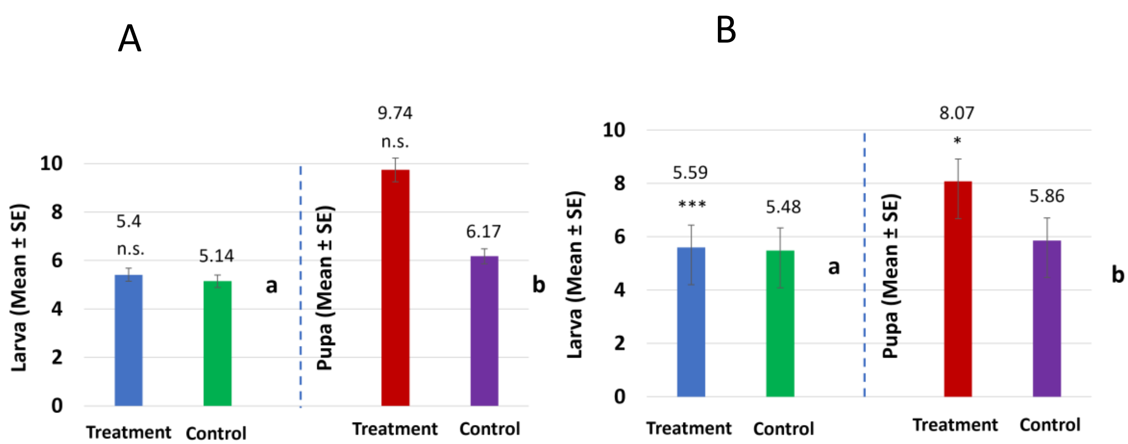

Figure 7. Mean ± SE of daily count of live (a) larvae and (b) pupae *Culex quinquefasciatus* (n=31). (A):10,000 µg/l glyphosate solution (treatment) versus water (control). Kolmogorov-Smirnov test: (a) P= 0.815, (b) P= 0.003 (a) Paired sample t-test: df= 30, t= 0.762, P= 0.452 (b) Mann Whitney U test: Z= 1.983, P< 0.051 (B): 100,000 µg/l glyphosate solution (treatment) vs water (control).Two-Sample Kolmogorov-Smirnov test: (a) Z= 0.396, P= 0.998; (b) Z= 2.064, P< 0.001 (a) Paired sample t-test: df= 25, t= 4.169, P< 0.001 (b) Mann Whitney U test: Z= 2.557, P< 0.05.). Asterisk on a bar indicates a significant difference at (*) 95% and (***) 99% confidence levels; n.s.= non-significant

Newly Emerged Adults (Mean)

The findings indicate that no significant difference was found in the number of emerged adults in the treatments and the control of both concentrations. The average number of emerged adults for the 10,000 µg/l glyphosate solution was 11.5 (Z= 0.671, P= 0.759; t-test: df= 9, t= 0.218, P=0.832) compared to its control with an average of 11.1 (Fig 8A). For the 100,000 µg/l glyphosate solution, the average was 12.9 (Z= 1.118, P=0.164; t-test: df= 9, t= 1.843, P= 0.098) compared to the control with an average of 9.4. (Fig 8B)

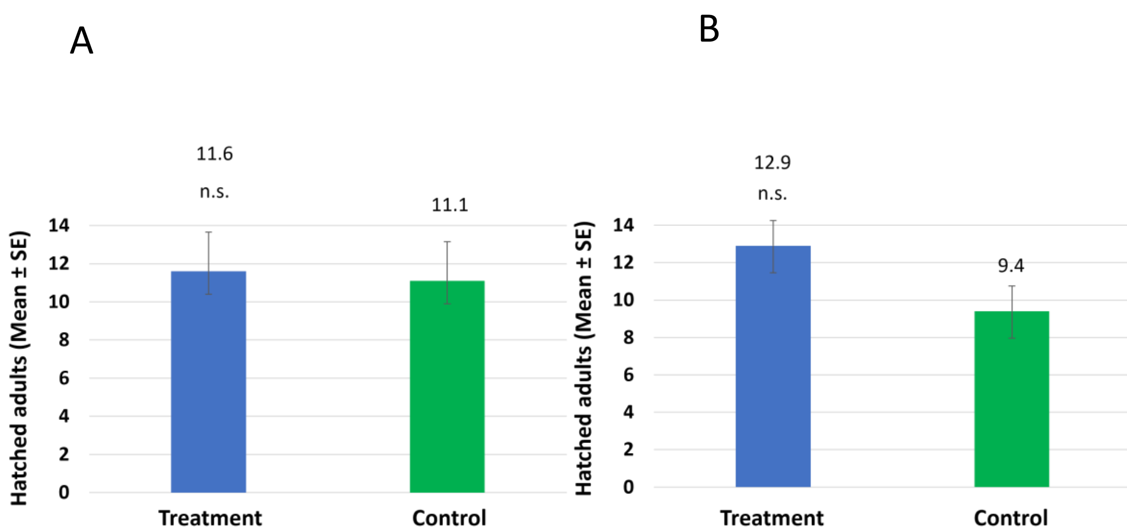

Figure 8. Hatched adults (Mean ± SE) *Culex quinquefasciatus*. (A): 10,000 µg/l glyphosate solution) versus control (water) Kolmogorov-Smirnov test (n=10): Z= 0.671, P= 0.759; t-test: df= 9, t= 0.218, P=0.832 (B): treatment (100,000 µg/l glyphosate solution) and control (water). Kolmogorov-Smirnov test (n=10): Z= 1.118, P=0.164; t-test: df= 9, t= 1.843, P= 0.098; n.s.= non-significant

Survival (Mean)

This study examined larval survival of *Culex quinquefasciatus* larvae over 30 days to determine whether the exposure to the 10,000 µg/l and 100,000 µg/l glyphosate solution created a statistically significant difference in the survival rate compared to the controls. In the first few days (0 to 4), the survival rates were similar between the two groups (Fig 9). From day 5 to day 7, the survival rate in the treatment was slightly higher than the control. By day 7, the treatment group with 10,000 µg/l of glyphosate showed higher survival rates (n= 19) than the control (n= 14) (Fig 9A). This trend reversed from day 8 to day 9, with the control group eventually showing a higher survival rate. The rate eventually became constant from day 10 to day 30. The same pattern was exhibited for the 100,000 µg/l with a constant rate from day 0 to 5 and day 9 (Fig 9B). From day 5 to 8 and day 10 to 17, the treatment group showed a higher survival rate than the control group. The survival rate eventually becomes relatively constant from day 18 to 25 (Fig 9B).

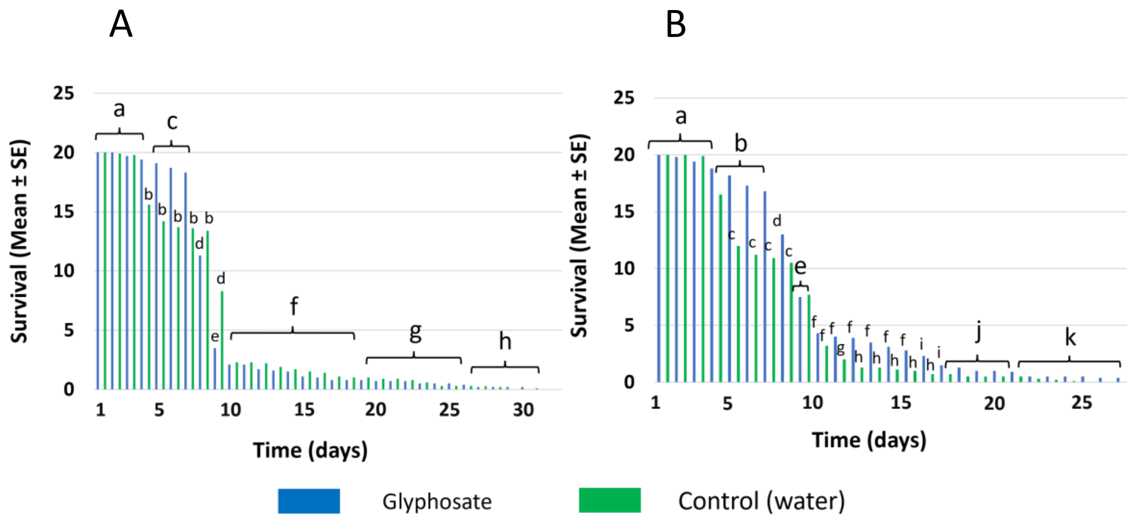

Figure 9. Effect of glyphosate (mean ± SEM) on the survival of the *Culex quinquefasciatus* larvae. (A): 10,000 µg/l glyphosate solution ANOVA (n= 58, df= 57, F= 149.395, P<0.001). (B):100,000 µg/l glyphosate ANOVA (n= 54, df= 53, F= 126.699, P<0.001). Different letters indicate statistically significant groups (Waller-Duncan post-hoc test).

Adult Body Length and Wingspan (Mean)

The length and wingspan of the adults developed from each group were measured using morphometric analysis. Tests of statistical significance (Kolmogorov-Smirnov Test and Paired sample t-test) were performed to see the impact of the two glyphosate concentrations on the body length and wingspan of the emerged adults.

For the 10,000 µg/l, the analysis revealed a significant difference (Z= 2.089, P<0.001) in the body length of the treated mosquitos, which appeared to be shorter compared to the control group with a 99.9% confidence level (Fig

10A). The wingspans, on the other hand, did not show any significant differences when both groups were compared (Z= 0.384, P>0.05) (Fig 10A).

For the 100,000 µg/l, the analysis revealed a non-significant difference in body length between the treated and the control mosquitos (Z= 0.485, P>0.05) (Fig 10B). They both exhibited about the same body length. On the other hand, the size of the treated mosquitoes' wingspan was more significant than that of the control group, with a 95% confidence level (P< 0.05) (Fig 10B).

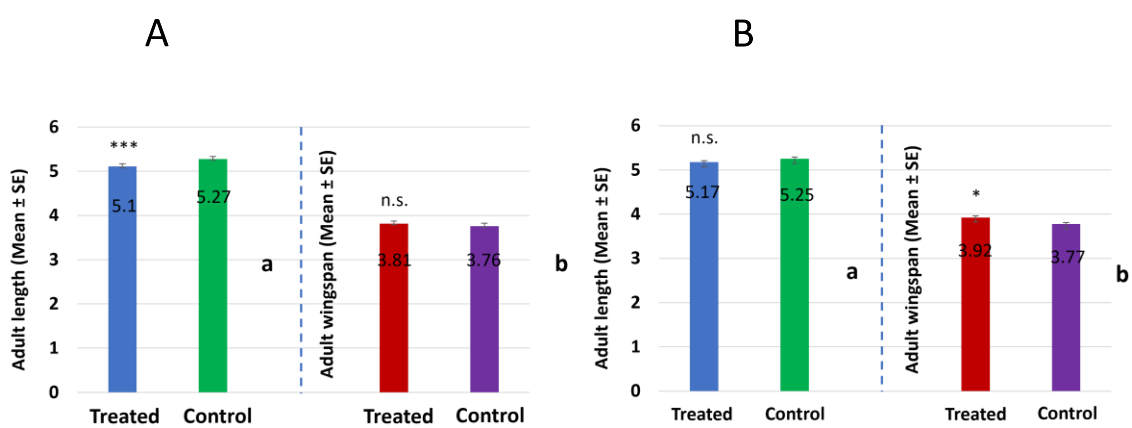

Figure 10. Mean ± SE of (a) adult length and (b) adult wingspan in mm for larvae treated with glyphosate versus control (A): 10,000 µg/l glyphosate versus control larvae in water. Kolmogorov-Smirnov Test, (a): Z= 2.089, P< 0.001; (b): Z= 0.384, P= 1.00. (a): Mann Whitney U test, Z= 3.384, P< 0.001; (b): Paired sample t-test, n= 66, df= 65, t= 1.017, P= 0.313. (B): 100,000 µg/l glyphosate versus control larvae in water. Kolmogorov-Smirnov Test, (a): Z= 0.485, P= 0.973; (b): Z= 1.213, P= 0.106. Paired sample t-test, n= 34, df= 33; (a): t= 0.653, P= 0.518; (b): t= 2.646, P< 0.05. An asterisk (*) on a bar indicates a significant difference at 95% confidence levels and (***) 99% confidence levels. n.s.= non-significant.

Oviposition

Mean number of egg rafts deposited in the treatment

The *Culex quinquefasciatus* females exposed to the 10,000 µg/l glyphosate solution showed no significant difference in the number of egg rafts deposited in the solution compared to the control group containing water (ANOVA: df= 3, 20; F= 7.399) (Fig 11). The mean number of egg rafts deposited in the 10,000 µg/l glyphosate solution was 5.3 (mean=5.3), and the mean number of egg rafts deposited in the control was 7.3 (mean=7.3). Statistically, there was no significant difference between those numbers (Waller-Duncan Posthoc test P> 0.05).

The *Culex quinquefasciatus* females exposed to the 100,000 µg/l glyphosate solution showed a significant difference in the number of egg rafts deposited in the solution compared to the control group with water (ANOVA: df= 3, 20; F= 7.399) (Fig 11). The mean number of egg rafts deposited in the 100,000 µg/l glyphosate solution was 4 (mean=4), and the mean number of egg rafts deposited in the control was 51.16 (mean=51.16). Statistically, those two numbers significantly differed at 99% confidence level (Waller-Duncan Posthoc test P<0.05).

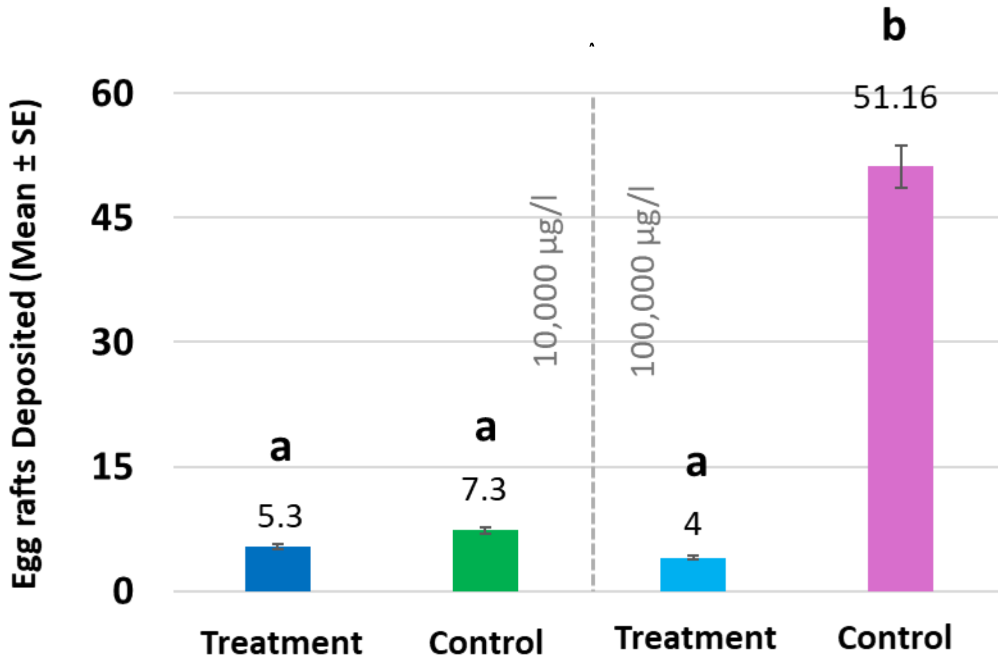

Figure 11. Mean ± SE of the egg rafts deposited by female *Culex quinquefasciatus* in a treatment medium with Glyphosate versus a control medium (water). n= 6. ANOVA: df= 3, 20; F= 7.399; P= 0.002, followed by a Waller-Duncan Posthoc test. Different letters on bars indicate a statistically significant difference. The mean value of each experimental group is shown on top of the bars.

Mean number of live eggs deposited in the treatment

The females of the *Culex quinquefasciatus* exposed to 10,000 µg/l glyphosate solution laid, on average, 375 live eggs compared to the control group who laid 457 live eggs (ANOVA: df= 3, 20; F= 5.663), which resulted in no significant difference between the number of eggs laid in the treatment and control group (P> 0.05) (Fig 12). Conversely, with the 100,000 µg/l glyphosate solution, the *Culex quinquefasciatus* females laid on average, 271 live eggs (ANOVA: df= 3, 20; F= 5.663) compared to the control group, where 4283 live eggs were laid Fig 12). These results show a significant difference between these two numbers with a 99% confidence level (P<0.05).

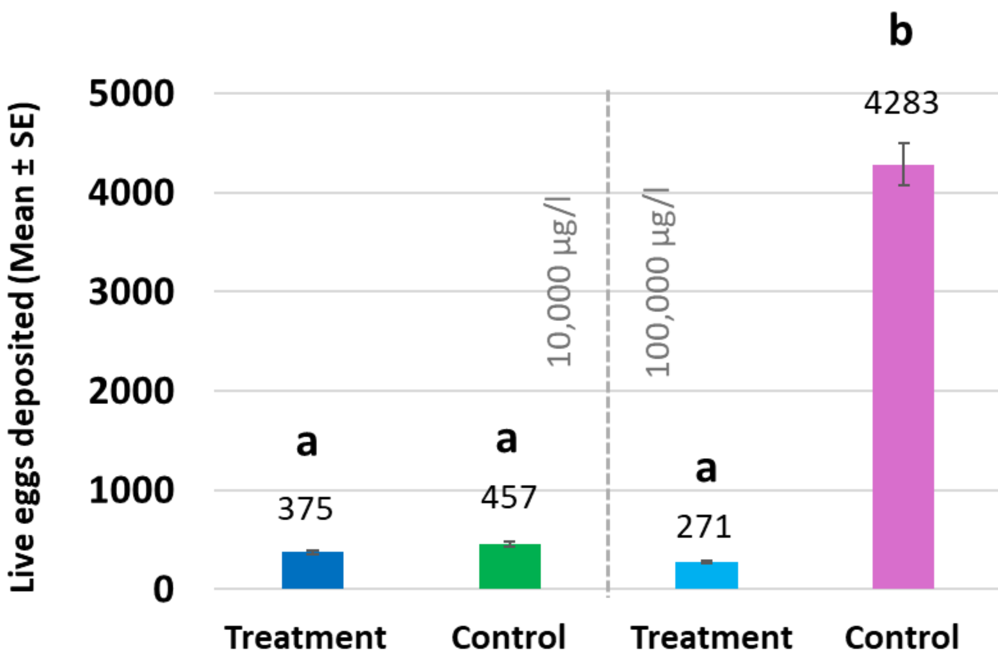

Figure 12. Mean ± SE of the eggs deposited by female *Culex quinquefasciatus* in a treatment medium with Glyphosate versus a control medium (water). n= 6. ANOVA: df= 3, 20; F= 5.663; P= 0.006, followed by a Waller-Duncan Posthoc test. Different letters on bars indicate a statistically significant difference. The mean value of each experimental group is shown on top of the bars.

<u>Mean number of first instar larvae hatched</u>

The first-instar larvae hatched in the 10,000 µg/l glyphosate solution had a mean number of 572, and the control group had a mean number of 642. The ANOVA followed by a Waller-Duncan Posthoc test revealed no significant difference between the two numbers (P>0.05) (Fig 13). On the other hand, the mean number of first-instar larvae hatched in the 100,000 µg/l glyphosate solution was 407 compared to the control, with 4,945 first instar. The results showed a significant difference with a 95% confidence level ($P<0.05$). (Fig 13).

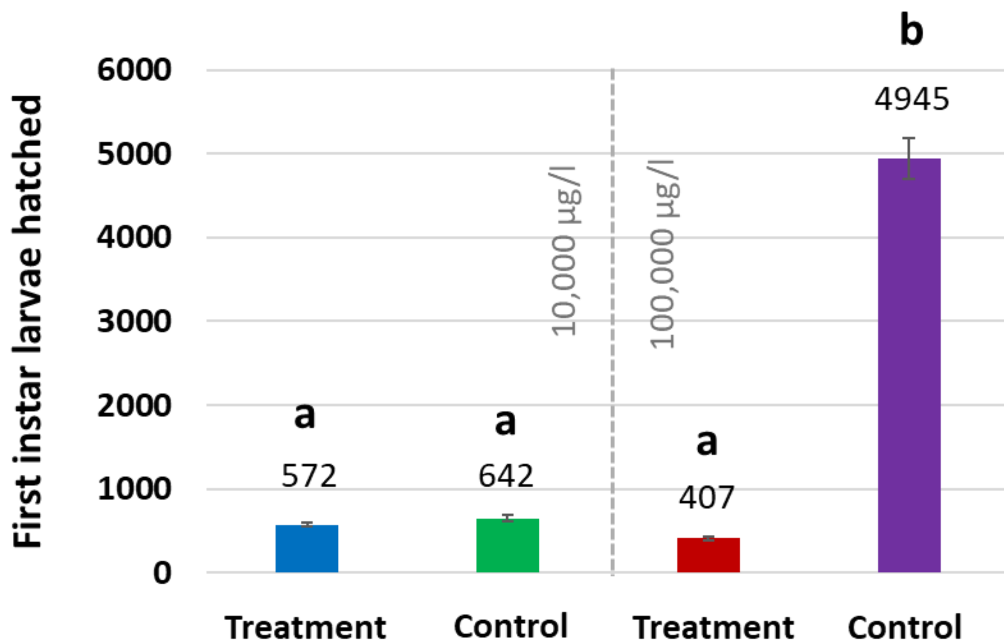

Figure 13. Mean ± SE of first instar larvae hatched in glyphosate solutions versus water (control) within the first 3 days of hatching eggs of *Culex quinquefasciatus*. n= 6. ANOVA: df= 3, 20; F= 5.970; P= 0.004, followed by a Waller-Duncan Posthoc test. Different letters on bars indicate a statistically significant difference. The mean value of each experimental group is shown on top of the bars.

Triple-Choice Assay

The Triple-Choice Assay experiment involved exposure of gravid females to three different media: the control group (Water), the 10,000 µg/l, and 100,000 µg/l glyphosate solutions. The results showed that, on average, the female laid 2.1 egg rafts in the 10,000 µg/l glyphosate solution, 0.1 in the 10,000 µg/l glyphosate solution, and 35 egg rafts in the control group. The ANOVA followed by a Waller-Duncan Posthoc test revealed a significant difference in the number of egg rafts laid in the two glyphosate solutions compared to the control group (df= 2, 12; F= 8.509, P<0.05) (Fig 14).

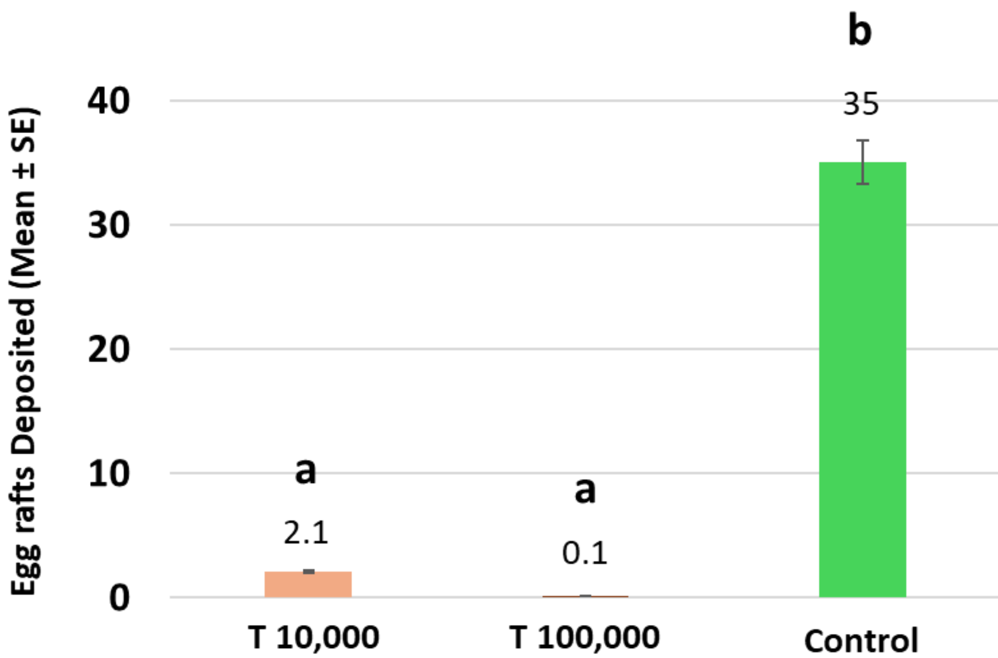

Figure 14. Mean ± SE of the egg rafts deposited by female *Culex quinquefasciatus* in a triple choice assay (10,000 and 100,000 µg/l Glyphosate) versus a control medium (water). n= 5. ANOVA: df= 2, 12; F= 8.509; P= 0.005, followed by a Waller-Duncan Posthoc test. Different letters on bars indicate a statistically significant difference. The mean value of each experimental group is shown on top of the bars.

Responding to the Research Questions

Effect of 10,000 µg/l or 100.000 µg/l of glyphosate solution on the larval development of *Culex quinquefasciatus*

The first research question addressed the impact of a 10,000 µg/l or 100.000 µg/l of glyphosate solution on the larval development of *Culex quinquefasciatus*. The result from the exposure to both glyphosate concentrations did not show a significant impact on the larval development of *Culex quinquefasciatus*.

Effect of 10,000 µg/l or 100,000 µg/l glyphosate solution on the adult emergence of *Culex quinquefasciatus*

The second research question examined the effects of the two glyphosate concentrations on the emergence of *Culex quinquefasciatus*. The results from this exposure indicated a non-significant difference between the treatment versus the control.

Effect of glyphosate concentrations on the oviposition and hatching rate of *Culex quinquefasciatus*

The third research question analyzed the effects of the two glyphosate concentrations on the oviposition and hatching rate of *Culex quinquefasciatus*. The study's findings demonstrate that the impact on the oviposition and the hatching rate was non-significant compared to the control when the 10,000 µg/l of glyphosate solution was used. On the other hand, the 100,000 µg/l of glyphosate had on the hatching rate and the oviposition, compared to the control group.

Effect of glyphosate concentrations on the fitness of *Culex quinquefasciatus*

This fourth research question focuses on how impactful glyphosate solutions were on the fitness of *culex quinquefasciatus.* The results showed an impact on the body length of the treated mosquitoes and a non-significant change in the wingspan compared to the control when exposed to the 10,000 µg/l. On the other hand, the exposure to the 100,000 µg/l showed a non-significant change in the body length, but the wingspans of the treated mosquitoes were more considerable compared to the control.

Chapter Summary

The results of this study provide evidence that exposure to glyphosate concentrations ranging from 10,000 to 100,000 µg/l has minimal effects on the survival, development, and adult emergence of *Culex quinquefasciatus* under controlled conditions. Despite variations in outcomes, no substantial differences were observed beyond a marginal increase in wingspan at the higher exposure level. These findings have implications for the risks associated with glyphosate exposure and the ecological balance of insect populations. These implications are discussed in the next chapter.

CHAPTER FIVE
DISCUSSION

This chapter interprets results obtained from the study of the effect of glyphosate solution concentrations of 10,000 µg/l and 100,000 µg/l on *Culex quinquefasciatus* compared to one another and a control group. First, the results are discussed in the context of previous studies and research literature. Considerations and implications are addressed, followed by strengths and limitations of the research and recommendations for both research and practice.

The Results in Context

The lack of consistent results concerning the impact of glyphosate on *Culex quinquefasciatus* is frustrating. However, it does not lead to easy answers concerning whether and when it should be used. To that end, exploring the discrepancies between this study and previous research and the broader ecological dynamics influenced by glyphosate provides context and positioning for the study results.

Environmental implications

There are ecological and environmental health implications of glyphosate usage (Beckie et al., 2020; Romano et al., 2021). Health implications concerning endocrinal disease, developmental and metabolic processes, and human health concerning glyphosate exposure continue to be investigated (Meftaul et al., 2020;

Romano et al., 2021). The agricultural systems today rely heavily on glyphosate for effective weed management that does not adversely affect crops (Beckie et al., 2020). This dependency highlights the challenge of finding sustainable alternatives that can balance agricultural productivity while minimizing ecological disruption and general risks to health (Beckie et al., 2020). While this study found that the 10,000 µg/l and 100,000 µg/l glyphosate concentrations do not significantly impact the survival or development of *Culex quinquefasciatus*, comparative analysis with prior research reveals a complex picture of glyphosate's interaction with mosquito populations and the cascading effects on the ecosystem health.

Studies reveal diverse responses of mosquito populations to glyphosate, suggesting variability based on species, developmental stages, and environmental conditions (Kibuthu et al., 2016; Nikbakhtzadeh & Fuentes, 2022). This variability underscores the importance of a systems-level approach to pesticide management, considering the differential impacts on non-target species and the broader ecological dynamics.

Analysis of Discrepancies

The discrepancies observed between this study and others relate to a research gap concerning the impacts of glyphosate on mosquitoes and environmental health. Nikbakhtzadeh and Fuentes (2022) did find negative impacts of glyphosate on oviposition and larval development. This may suggest that glyphosate's effects on mosquito populations involve factorial and behavioral changes that were not captured as variables, or the discrepancy could be attributed to different experimental conditions. The threshold of variables in this study were larval development, survival rates, oviposition, and fitness. In contrast, the study by Nikbakhtzadeh and Fuentes (2022) looked at sub-lethal characteristics such as developmental outcomes and behavioral changes.

Alignment of Findings with Previous Studies

Even at the higher concentration of 100,000 µg/l, no significant effects were noted on larval survival or adult emergence in this study. This aligns with the observations of Kibuthu et al. (2016), who noted the complex interactions between agricultural chemicals and mosquito life history traits. This study confirms findings in previous research that even at higher concentrations, glyphosate has limited lethal effects on *Culex quinquefasciatus*, warranting further investigation into sub-lethal impacts (Kibuthu et al., 2016).

Sub-lethal Effects of Glyphosate

The sub-lethal effects observed in related studies have included altered behavior and developmental delays (Bara et al., 2014; Baglan et al., 2018). While these may have only indirect impacts on the survival of mosquitoes, they may highlight further concerns about the broad effects on health and the ecological implications concerning the use of glyphosate. Considering the other literature, like the studies conducted by Judd (2018) on pesticides' impact on reproductive biology and Khan et al. (2022) on the predatory efficiency of Odonata nymphs, it is evident that glyphosate's impact extends beyond direct mortality. There is the potential for glyphosate to induce sub-lethal effects relating to development and behavior in mosquitoes, which will have cascading effects on the vector’s competence and disease transmission (Bara et al., 2014; Bataillard et al., 2020). While direct mortality may not be significantly affected at the concentrations tested, the possibility of subtler impacts warrants further investigation.

Oviposition

The effect of the glyphosate solutions on oviposition showed remarkable results. When the gravid females were exposed to 10,000 µg/l of glyphosate, they laid on average 5.3 egg rafts, compared to the control, where the females laid around 7.3 egg rafts. The 100,000 µg/l glyphosate solution showed even fewer egg rafts deposited than the control, with 51.16. These results indicate that the gravid females prefer the aged top water to lay their eggs. Other studies have shown that mosquitos use their sense of taste and vision to find the appropriate site for laying their eggs (Day, 2016).

Null Results Require Further Investigation

The lack of significant differences in survival rates between the two concentrations suggests a threshold effect where glyphosate, within the tested range, does not exhibit a direct toxic impact on *Culex quinquefasciatus*. This impacts the environmental safety profile of glyphosate and its widespread use (Atwood & Paisley-Jones, 2017). The financial and economic analyses of mosquito-borne disease outbreaks highlight the significant costs associated with vector control and disease management and the sustainability and efficacy of public health strategies involving pesticides (Kastner et al., 2017). To that end, a comprehensive mapping of impacts, secondary effects, and dynamics is needed.

Summary of Discussion

The study's findings reveal minimal direct toxicity of glyphosate on *Culex quinquefasciatus*, corroborating the herbicide's selective mechanism of action and its comparatively lower toxicity to animals. However, the observed slight variations in adult morphological characteristics beckon further investigation into potential sub-lethal effects or delayed impacts not captured within this study's framework. The nuanced sub-lethal impacts, including potential increases in susceptibility to infections, underscore the importance of a holistic evaluation of glyphosate's effects on mosquito populations and, by extension, on vector-borne disease dynamics.

Strengths and Limitations

Strengths

This research study used a rigorous experimental design that allowed easy replication to evaluate the effects of glyphosate on *Culex quinquefasciatus*, using different concentrations of glyphosate and a control group for comparison provided insights regarding how and if glyphosate has a negative effect on mosquito populations. The research design considered a broad spectrum of outcome measures, including survival rates, developmental progression, and morphological changes. By not limiting the investigation to a single aspect of mosquito biology, the study offers a multifaceted perspective on the potential consequences of glyphosate exposure.

Limitations

The study was limited to two concentrations, which may not reflect the full range of concentrations used in natural settings. The relatively small sample sizes can impair the statistical significance of the results, making it harder to detect more subtle effects. While focusing on direct survival, development, and morphology, the study did not measure other potential impacts, such as behavioral changes, which could have significant indirect implications for survival. The laboratory conditions under which the study was conducted cannot replicate the complex dynamics in natural environments, limiting the generalizability of the findings.

Recommendations for Research and Practice

Recommendations for Research

More research is required to understand the effect of glyphosate and the mechanisms through which it changes the traits of mosquitoes that are important to their pathogen transmission, behavior, and life cycle. Klátyik et al. (2023). The ecotoxicity of glyphosate suggests that its broad ecological impacts, including potential effects on non-target species, require a thorough investigation (Klátyik et al., 2023; Talyn et al., 2023). It is, therefore, not enough to conduct laboratory-based research with limited field realistic dynamics.

Recommendations for Practice

The minimal impact of the tested doses of glyphosate on mosquitoes suggests that alternative, environmentally friendly methods can effectively manage mosquito populations without adverse ecological effects. The study's findings highlight the importance of understanding glyphosate's ecological impacts, requiring ongoing surveillance and evaluating efforts to inform and develop public health strategies.

Promoting evidence-based practice. There is also a need to review and adjust regulations on glyphosate use based on current research findings. Realigning protocols in practice with the latest evidence can improve outcomes about the goals of glyphosate usage on the mosquito population and human health.

Potential for cascading effects. The absence of significant effects from both concentrations of glyphosate on *Culex quinquefasciatus* suggests that glyphosate, primarily targeting the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme in plants, may have limited direct toxicity to non-target aquatic insects such as mosquitoes. This aligns with the specific action mechanism of glyphosate and its relatively lower toxicity to animals. However, the slight variations observed in adult morphological characteristics warrant further investigation to explore potential sub-lethal effects or delayed impacts not captured within the scope of this study. The less visible sub-lethal impacts of glyphosate include the susceptibility of insects to infection, possibly by inhibiting myelinization (Klátyik et al., 2023; Nation, 2022; Smith et al., 2021). Given the role of *Culex quinquefasciatus* as a vector for diseases such as West Nile virus, understanding how environmental pollutants like glyphosate affect mosquito populations and whether they may increase the potential for vector-borne disease even with survival-reducing impacts is crucial for integrated evaluation of outcomes in public environmental health initiatives.

Expansion of Research through Advocacy. Advocating for research funding on glyphosate's broader ecological impacts can expand the current research efforts, increase the resolution of those findings, and support more significant levels of collaboration. This could help address the research problem of glyphosate's sub-lethal effects while informing agricultural management and public health policy. Fostering collaboration between sectors to share best practices and information on glyphosate usage and mosquito control can be effective. Initiatives could include developing summary materials regarding research evidence and best practices, with educational materials on alternative methods promoting safer environmental practices and informed glyphosate use and concentration decision-making.

CONCLUSION

This study evaluated the effects of glyphosate at 10,000 µg/l and 100,000 µg/l and determined the survival rates, emergence, and morphological characteristics of *Culex quinquefasciatus*. The findings indicated that glyphosate, at these concentrations, has a limited impact on the lifecycle and physical development of *Culex quinquefasciatus*. At the lower concentration of 10,000 µg/l, glyphosate showed negligible effects on the mosquitoes' survival, emergence, and morphology. Similarly, at 100,000 µg/l, there was a slight increase in the average count of hatched adults. The comparison between the two concentrations revealed minimal differences, suggesting that glyphosate's impact on *Culex quinquefasciatus* is limited across the tested range. These results are significant for environmental management, indicating that glyphosate, at concentrations commonly used in vector control, may not adversely affect *Culex quinquefasciatus* populations. However, the study underscores the importance of further research to explore potential sub-lethal effects and broader ecological impacts of glyphosate, particularly on non-target species and ecosystem health. The findings advocate for a balanced approach in practice as well, which is a complicated request given the various sectors, occupations, and livelihoods involved in the use and efficacy of glyphosate and its potential risk.

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