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ABSTRACT

The production and use of mosquito insecticides has long been practiced; presently, the search for natural insecticides and larvicides is a flourishing field. Natural insecticides and larvicides persist less in the environment and are less toxic than synthetic insecticides such as DDT (dichloro-diphenyl-trichloroethane). I studied the effectiveness of citronella grass, *Cymbopogon nardus*, as a larvicide against *Culex quinquefasciatus* and *Culex nigripalpus* mosquito larvae and how it affected their behaviors. The larvae were exposed to three different treatments: two self-prepared extractions of citronella oil of different concentrations and a natural commercialized citronella repellant spray. I recorded the number of deaths and behavioral changes before and after the treatment twice a day. Results showed that larvae mortality between the control group and the three treatments were significantly different. The two citronella oil extraction treatments collectively killed an average of 52% of the larvae and the commercial spray killed 100% of the larvae. The treatments also induced the larvae exposed to the citronella oil to progress in the life cycle more rapidly than the larvae in the control group. The larvae exhibited new behaviors after exposure to the citronella essential oil as well. The sudden behavior change after adding the citronella essential oil is key evidence that the citronella oil is immediately affecting the larvae. The results I obtained from this study suggest that the leaf and aerial parts essential oils of *C. nardus* are potential natural larvicides against *C. quinquefasciatus* and *C. nigripalpus* larvae.

La eficacia de extracto de aceite esencial de citronela como un larvicida contra *Culex* spp.

RESUMEN

La producción y uso de insecticidas contra mosquitos se ha practicado por mucho tiempo. Hoy en día, la búsqueda de insecticidas naturales y larvicidas es un campo de estudio en crecimiento. Los insecticidas y larvicidas naturales persisten menos en el medio ambiente y son menos tóxicos que los insecticidas sintéticos como DDT (dicloro-difenil-tricloroetano). Estudié la efectividad de la planta de citronela, *Cymbopogon nardus*, al ser usada como larvicida contra larvas de *Culex quinquefasciatus* y *Culex nigripalpus* y cómo afectaban sus comportamientos. Los resultados mostraron que la mortalidad de larvas entre el grupo control y los tres tratamientos fue significativamente diferente. Los dos tratamientos de extracción de aceite de citronela mataron colectivamente un promedio del 52% de las larvas y el spray comercial mató el 100%. Las larvas mostraron nuevos comportamientos después de la exposición al aceite de citronela. El cambio repentino de comportamiento después de agregar el aceite de citronela es evidencia de que este está afectando inmediatamente a las larvas. Los resultados que obtuve en este estudio sugieren que los aceites esenciales de las hojas de *C. nardus* son larvicidas naturales potenciales contra larvas de *C. quinquefasciatus* y *C. nigripalpus*.

Insect-transmitted diseases continue to afflict human populations. Mosquitos are the main vector for diseases such as malaria, filariases, yellow fever, dengue, and West Nile virus (WNV) (Fradin and Day, 2002). Dengue, in addition to Chikunguna and Zika, is one of the most significant mosquito-borne diseases in Costa Rica; all three of the diseases are transmitted by *Aedes aegypti* (ECDC, 2016). Several species of mosquitos are found in Costa Rica with multiple species from each of the following genera including *Aedes*, *Anopheles*, *Culex*, *Haemagogus*, and *Toxorhynchites* (Janzen, 1983). In addition to the *Aedes* genera, *Anopheles* mosquitoes can transmit malaria; *Culex* species can spread WNV, St. Louis encephalitis (SLEV), and Japanese encephalitis; and *Haemagogus* can transmit yellow fever (Sinka et al. 2010; CDC 2000; Cardosa et al. 2010). Mosquitoes are holometabolus insects part of the Culicidae family; only adult mosquitoes can act as vectors (Janzen, 1983). The larvae of mosquitoes are aquatic and require stagnant or slow moving water (Troyo et al., 2008). Thus, mosquito larvae must be approached using different tactics than adult mosquitoes.

The use of larvicides is one of several methods used to prevent and reduce the transmission of mosquito-borne diseases. By targeting aquatic breeding sites of mosquitoes, people aim to reduce the transmission of vector-borne diseases through Larval Source Management (LSM) (WHO, 2012). This entails killing mosquito larvae before they can reach adulthood and become vectors by regularly applying chemical or biological agents to their breeding site. However, synthetic larvicides have also been shown to have adverse effects on the environment and human health. Synthetic larvicides are non-biodegradable and can result in the development of resistance in mosquito vector species as well as biological magnification of toxic compounds via the food chain (Ghosh et al., 2012). Thus, the push to find and use effective natural insecticides by scientists and the public is gaining traction. Citronella grass, or *Cymbopogon nardus*, is a known natural insect repellant and part of the Poaceae family. Citronella grass contains the components citronellal, citronellol, geraniol, citral, α pinene, and limonene (Maia and Moore, 2011). The citronella oil extracted from *C. nardus* was shown to be an effective mosquito repellent on human subjects for up to two hours (Trongtokit et al., 2005). Citronella insect repellant products do not kill the adult insects they target. The components of citronella leaf extract could cause larval death, however, because they inhibit the respiratory function of the larvae (Widawati and Riandi, 2013). The respiration of the larvae could be obstructed by the thin oil film from the citronella essential oil that forms on the surface of the water. This layer of oil could block the siphon, the anatomical structure at the abdomen that larvae use to breathe (Kanis et al., 2012). There have been no published studies to date that have analyzed the effects of citronella essential oil on other aquatic organisms or the environment. There are relatively few studies that have been carried out to determine the efficacy of the citronella oil on larval mortality. There have been no studies that examine citronella essential oil's effect on larval behavior. In this study, I address the question: Is citronella essential oil an effective mosquito larvicide and does it change larval behavior? I predict that the citronella oil will induce death of the mosquito larvae and that it will affect the behavior of the larvae.

MATERIALS AND METHODS

Experimental Set-up:

The experiment was carried out over a course of 12 days from 9 May to 20 May 2017 in Monteverde. I used three different treatments and a control to study the effects of citronella oil as a larvicide of mosquitos. I collected *C. quinquefasciatus* and *C. nigripalpus* fourth instar larvae, which is the last mosquito larval stage before the pupa stage (Fig. 1). I collected the larvae from two study sites. The first site was in Monteverde located next to the Estación Biológica and contained the *C. quinquefasciatus* larvae, the other breeding site was in Cerro Plano and contained the *C. nigripalpus* larvae. There were 8 sample jars per treatment with ten larvae per sample, I collected 320 larvae in total. The sample jars mimicked a small-scale pond. Each sample jar contained 80 ml of pond water and 2 mm of soil from the pond in each container to provide the larvae with nutrients. This ensured that if any larvae died it was from the citronella oil rather than lack of food. I stored the larvae at the Instituto de Monteverde and observed them there.

I made two ethanolic citronella oil extracts of unknown concentrations using simple distillation. I ground 13 g of dried *C. nardus* leaves and aerial parts into a fine powder using a blender. Each extraction contained 6.5 grams of powder soaked in the appropriate amount of ethanol for 24 hours. The first extraction was one part *C. nardus* powder to two parts ethanol. The second extraction used a one to one ratio of *C. nardus* powder to ethanol. I then used a distillation apparatus and condenser to boil the respective solutions and extract the citronella oil.

In the first treatment, referred to as "extraction one," I added 20 drops of the first extraction of citronella oil to each sample. In the second treatment, referred to as "extraction two," I added 20 drops to each sample. In the third treatment, referred to as "commercial," I applied three sprays of a natural citronella insect repellent product to each sample. The concentration of the citronella essential oil in the commercial spray was also unknown. I added nothing to the control.

I first collected data on 11 May 2017. I observed the larval behavior before and immediately after the citronella was added, as well as in the long term: 2 hours after, 24 hours after, and twice a day until no larvae remained or until the end of the data collection period of 11 days. I applied each treatment only once per sample and immediately mixed the water in each sample jar for 15 seconds afterwards. During these observations, I tallied the number of larval deaths for each treatment. Mosquito larvae normally float at the water's surface when resting. Based on preliminary observations, I categorized the larval behaviors into four different, non-mutually exclusive categories: float, clump, twitch, and clean. I classified the "float" behavior as a larva resting at the water's surface. I classified the "clump" behavior as two or more larvae pressed next to each other. I classified the "twitch" behavior as a larva twitching its thorax and abdomen at the water's surface. I classified the "clean" behavior as a larva curled up to clean its siphon. Further examining larval behavior, once I applied the treatment I recorded and classified the "response time" as the amount of time it took for half of the mosquito larvae in each sample to float on the surface of the water after a disturbance. The disturbance was created by stirring a pipette three times within a sample jar; I did this for the three treatments and the control. I also monitored the life stage progression of the larvae and recorded the number of larvae in each of the treatments that progressed into the pupa or adult life stage.

A)

B)

Figure 1. The two species of Culicidae mosquitoes used in the experiments: A) *Culex quinquefasciatus* B) *Culex nigripalpus*. The siphon is the long tubular structure protruding from the abdomen; this body part helps the larvae breath. Mosquitoes identified using an online project gallery specific to *Culex* species identification: http://wrbu.si.edu/mqID/mq_cx_menu.html

Statistical Analyses and Calculations:

I used the Chi-squared test for larvae mortality and life stage progression. I calculated the average and standard deviation in regards to the response time of the larvae.

RESULTS

In the nine days of larvae collection, I gathered 320 mosquito larvae with 160 larvae of *C. quinquefasciatus* and *C. nigripalpus* each. Almost all the larvae (78 or 98%) in the control group survived compared to approximately half in the extraction one (38) and extraction two (39) treatments and none of the larvae survived in the commercial treatment (0). There was a highly significant difference in larvae mortality between the control group and the three treatments (χ^2 = 152.28, d.f. = 3, p<0.0001, Fig. 2). The average number of larvae killed in extraction one and extraction two was over half of the sample size (avg. mortality = 41.5 larvae, 52%). Almost all the larvae in the commercial treatment (79, 98.75%) died within 2 hours of being exposed to the citronella spray. The extraction one and extraction two treatment showed that approximately 10 larvae from each group died within 2 hours of exposure to the citronella oil and plateaued at approximately 40 larvae (50%) on the fourth day (Fig. 3).

There were significant differences in the proportion of larvae that were able to reach the pupae and adult mosquito life cycle stage between the treatments and the control (χ^2 = 24.97, d.f. = 3, p<0.0001, Fig. 4). Of the surviving larvae in the extraction one treatment, over half of the larvae progressed into the pupae or adult mosquito life form. For the extraction two treatment, 18 of the 39 remaining larvae reached the pupae or adult life stage. As for the control, only 22 of the 78 larvae, less than a third of the sample size, turned into pupae or adult mosquitoes.

The data regarding the response times of the larvae to float back to surface after a disturbance varied between the control (avg. time = 9.52 seconds ±9.28 SD), extraction one (21.99 s ±20.75 SD), extraction two (21.27 sec ±14.49 SD), and commercial groups (48.55 sec ±38.28 SD) (Fig. 5). Observations of larvae behavior revealed that the larvae exposed to the citronella extracts and the commercial spray displayed two new behaviors, “clean” and “twitch”, in addition to the behaviors “clump” and “float” that the control larvae exhibited (Fig. 6).

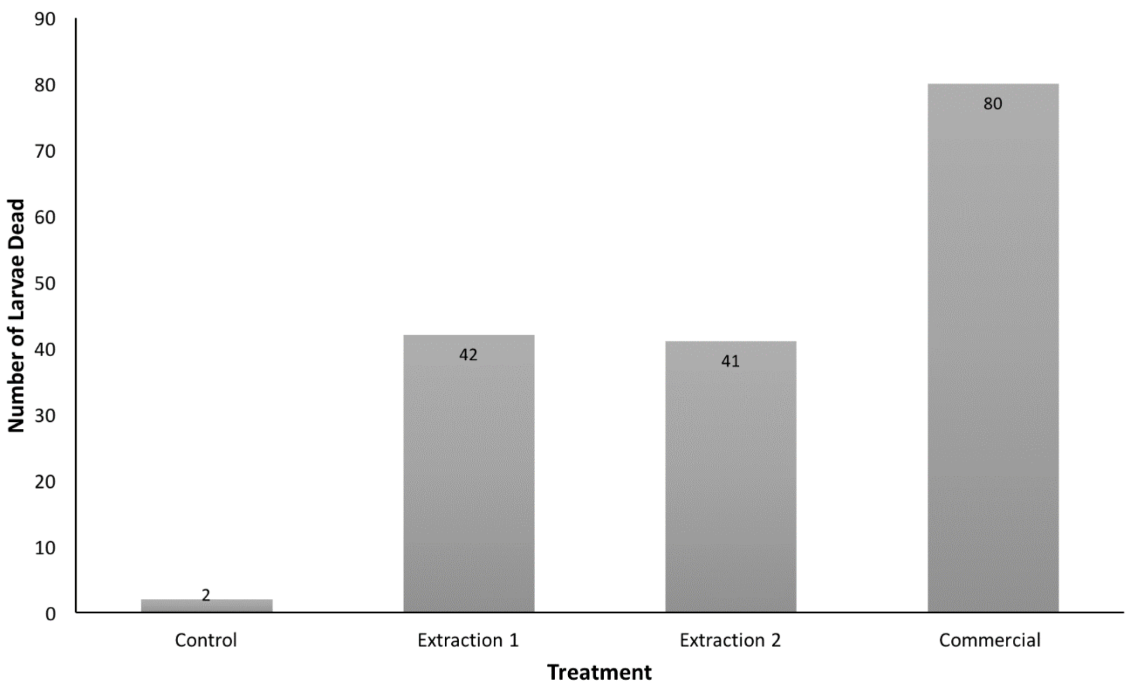

Figure 2. Larval mortality of *C. quinquefasciatus* and *C. nigripalpus* combined over a period of 11 days between the three treatments and the control. Sample size n=80 per treatment.

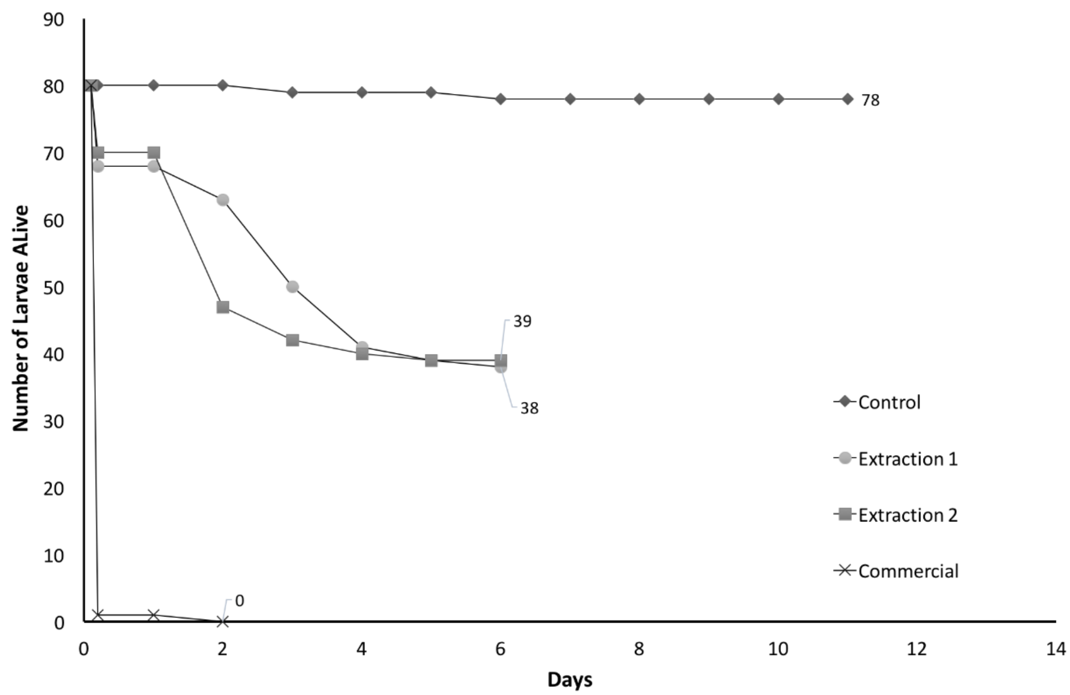

Figure 3. A daily comparison of the survivorship of larvae between the three treatments and the control over an 11-day period. The larvae in the control treatment experienced little larvae mortality (2.5%). Approximately half of the larvae in the extraction 1 (51.25%) and extraction 2 (52.5%) treatment died. The larvae in the commercial treatment experienced high larvae mortality (98.75%) within two hours of exposure to the citronella repellent.

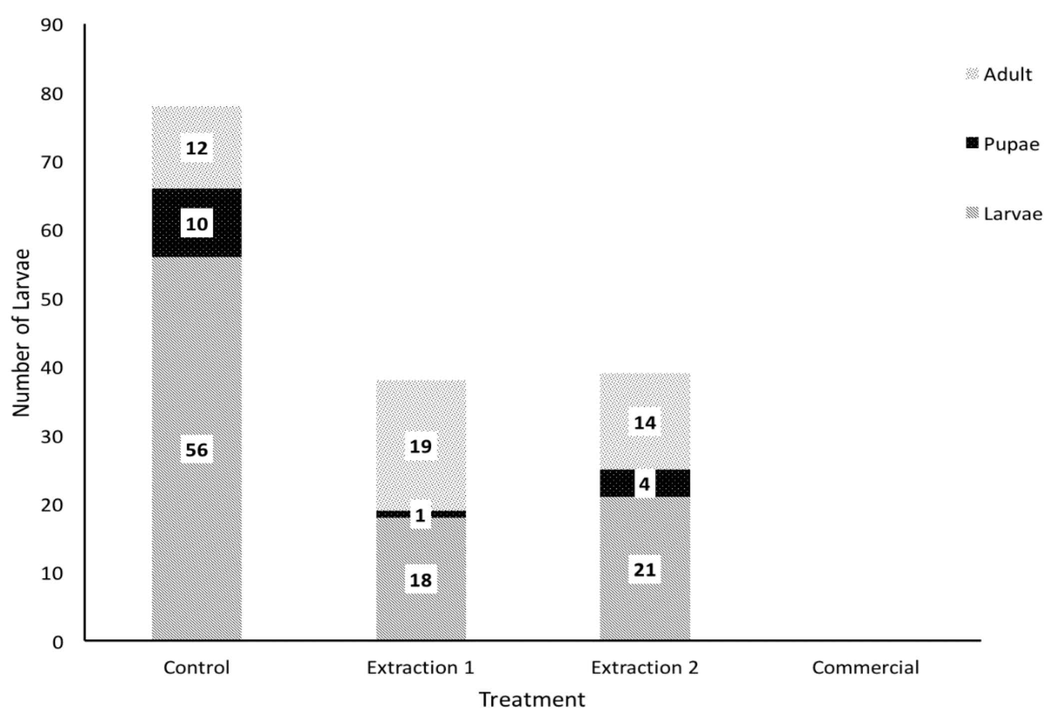

Figure 4. A progression of the life stages of the larvae of the total that survived the treatments from each treatment and the control in an 11-day period. Of the live larvae in the control, 28.2% of the larvae transitioned into the pupae or adult stage. Of the larvae that survived in the extraction one treatment, 52.6% of the larvae progressed into the pupae or adult stage. Of the surviving larvae in the extraction two treatment, 46.2% of the larvae became pupa or adults. None of the larvae in the commercial treatment survived long enough to progress into another life stage.

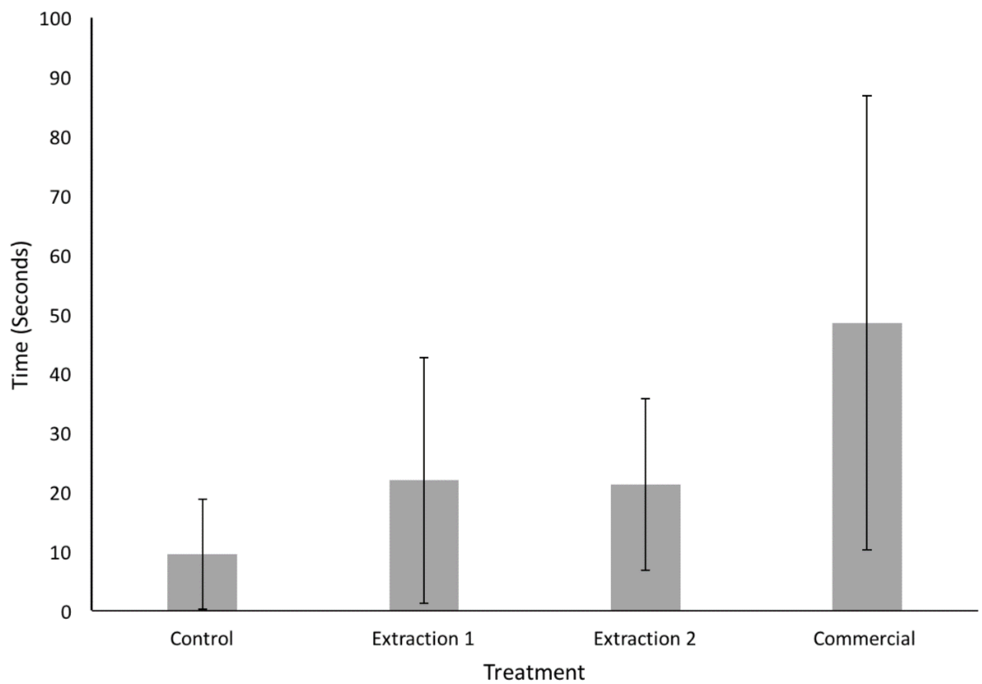

Figure 5. The average response time for larvae to float back up to the surface after a disturbance for the control, extraction 1, extraction 2, and the commercial treatment. The larvae in the commercial treatment took over five times as long on average to float back up to the surface compared to the control. The larvae in the extraction one and extraction two treatments took approximately twice as long to respond after a disturbance compared to the larvae in the control treatment.

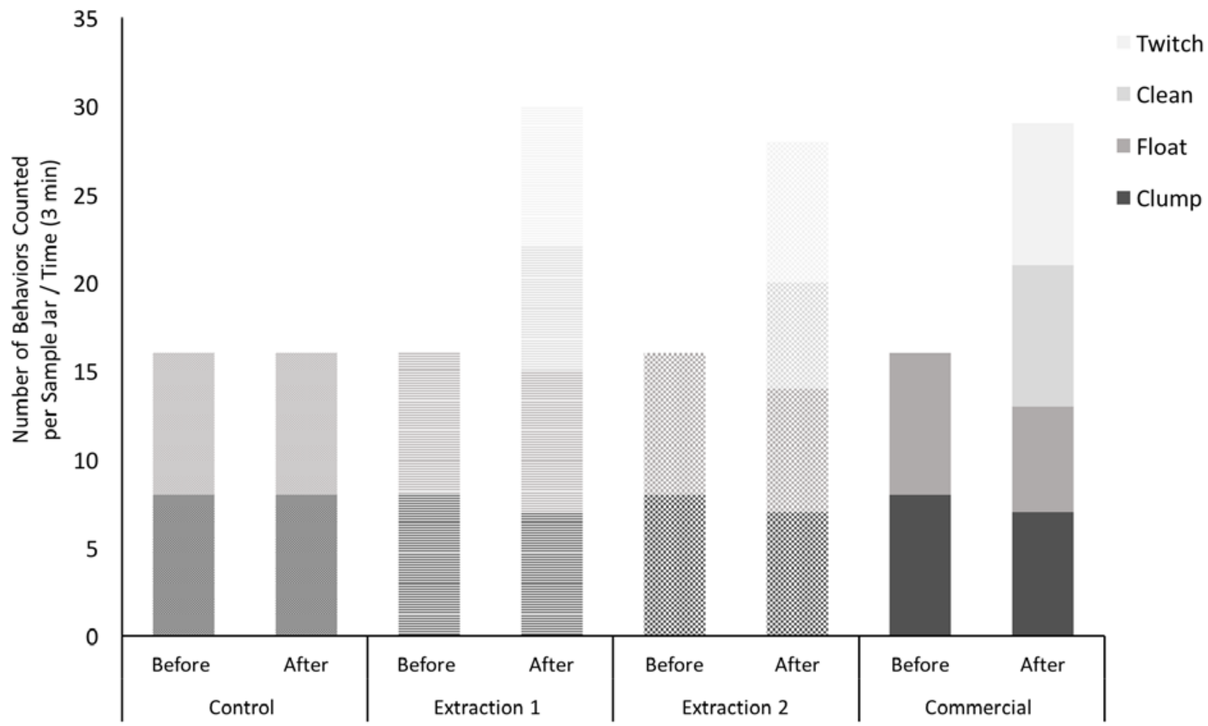

Figure 6. Observations of various larvae behavior per sample jar in three-minute time frames immediately before and after each treatment was applied. The larvae in the control sample jars only exhibited the "clump" and "float" behavior. The sample jars with larvae exposed to citronella in each of the three treatments exhibited the new behaviors "clean" and "twitch" in addition to "clump" and "float."

DISCUSSION

My results showed that citronella extract does have a significant effect on larvae mortality. The overall effectiveness of citronella oil as a larvicide (52%) was less than that found with citronella microcapsules tested on *Aedes aegypti* (99%) by Widawati and Riandi (2013). This inconsistency could be attributed to differences within mosquito genera or the methods used to expose the larvae to the active compounds of *C. nardus*. I was unable to determine the concentration of citronella in neither of the self-prepared essential oil extractions nor in the natural commercial citronella insect repellant spray. The discrepancy in the effectiveness of citronella essential oil as a larvicide could possibly be attributed to the study by Widawati and Riandi (2013) using a higher concentration of citronella in the microcapsules. More research should be conducted on the effects citronella oil has on different mosquito genera as well as what concentration of citronella essential oil is optimum as a larvicide.

The commercial spray killed all the larvae suggesting that the combination of multiple active compounds from different plants create a more effective larvicide than the active ingredients from any one plant. The commercial citronella repellent spray contained distilled water, citronella oil extract from *C. nardus*, lemon oil extract from *Citrus limonum*, cetostearyl alcohol, and polyethylene glycol 400, glycerin USP, peppermint oil extract, copolymers of N-Vinylpyrrolidone and fatty acid a-olefins, carbo-mer, Triethanolamine, Butylated hydroxytoluene, Benzyl alcohol, Benzoic acid, and sorbic acid. Peppermint oil extracted from *Mentha piperita* exhibited LC_{50} and LC_{90} values of 111.9 and 295.18 ppm against *A. aegypti* exhibiting effective larvicidal properties (Kumar et al., 2011). Lemon oil extract from *Cupressus arizonica* showed almost a 100% mortality against *Anopheles stephensi* (Sedaghat et al., 2011). The citronella, peppermint, and lemon essential oils in conjunction with the alcohol and acidic compounds presented in the spray resulted in a more effective larvicide. Further research should be done to examine the interactions between different essential oils and their combined effects on larvae survival. This can further improve methodologies and the products used to manufacture larvicides.

The significant number of larvae that progressed into the pupae or adult mosquito life stage in the extraction one and extraction two treatment suggest that the citronella oil provoked an emergency response within the larvae. Mosquito larvae typically stay in the larval stage for a minimum of four days before transitioning in to the pupa stage (CDC, 2012). Most of the larvae in the extraction one and extraction two treatments progressed into the pupa stage in less than four days after being collected. Larvae in the control group collected on the same day from the same breeding site did not show the same increased rate of progression within the life stages as the larvae in the extraction one and extraction two treatments. Other studies that analyze insect life-history found that rate of progression through the life stages deviated from the norm in response to environmental stresses (Frago and Bauce 2014; Inbar et al. 2001). More research should be conducted examining specifically the life-history progression of mosquitoes in response to stress induced by larvicides to gain a better understanding of the specific effects the larvicide have on mosquitoes.

The average response times of the larvae returning to the surface of the water after a disturbance correlated with the mortality in each treatment. The larvae in the commercial treatment had the highest mortality count (80 larvae) as well as the longest average response times (48.5 seconds) compared to the extraction treatments in which approximately half of the larvae died and the response time was approximately 21.5 seconds for each. The larvae in the control showed both the lowest mortality count (2 larvae) and the least amount of time needed to respond (9.5 seconds). These results suggest that the longer the average response time to float up to the surface the larvae exhibited, the more affected they were. This implies that the citronella compound not only affected the survival of the *C. quinquefasciatus* and *C. nigripalpus* larvae but also the behavioral characteristics of both species.

Observations of larval behavior before and after exposure to the citronella compounds revealed two new behaviors of the larvae not displayed in the control group; these behaviors were “clean” and “twitch.” The “clean” and “twitch” behaviors suggest that the citronella oil stuck to the larvae’s bodies and that the larvae were moving in this manner to clean themselves. As a study done with the oleoresin of *Copaifera* as a larvicide against *Aedes aegypti* suggests, the oil film formed on the surface of the sample jars could have been ingested by the larvae through its siphon (Kannis et al., 2012). This may further explain the larvae’s increased display of the “clean” behavior, however, more research is needed to verify this explanation.

A study that used essential oil from *Schinus terebinthifolia,* the Brazilian pepper tree, against *Stegomyia aegypti*, a mosquito vector of dengue found that the exoskeleton of the larvae lost structural integrity and that some internal organs were directly damaged (Pratti et al., 2015). In instances where larvae died within an hour of exposure, the researchers noted that the larvae died because they could not determine how to orient their bodies upward to breath (Pratti et al., 2015). Both the citronella essential oil and the essential oil of *S. terebinthifolia* contain α pinene as a major compound; this compound could have a role in deteriorating the larvae's bodies. The results from the study conducted by Pratti (2015) suggest that citronella essential oil potentially killed the larvae by degrading their exoskeleton. However, further research is needed to examine what the exact cause of death is and the role each of the active compounds in the essential oil played. This can help further hone future larvicidal products and methodologies.

Culex species are regarded as primary vectors of West Nile Virus (WNV) and St. Louis Encephalitis (SLEV), viruses of the *Flavivirdae* family (CDC, 2000). Although WNV and SLEV are not prevalent in Costa Rica, these encephalitis viruses are established in areas from North America to Venezuela (WHO, 2011). Laboratory testing and field evidence show *C. nigripalpus* to be the main vectors of WNV in Louisiana and Florida, and SLEV in Florida as well (Godsey et al. 2013, Vitek et al. 2008). The behavior of *C. nigripalpus*, such as its generalist feeding behavior, suggests vector competence for arboviruses (Vitek et al., 2008). *C. quinquefasciatus* plays a key role as an enzootic vector, it feeds from both birds and humans (Godsey et al., 2013). This behavior makes *C. quinquefasciatus* a potential vector of WNV should the disease become common in Costa Rica (Troyo et al., 2008). *A. aegypti* is the most common species found in the Greater Puntarenas area of Costa Rica which includes the study sites of Monteverde and Cerro Plano used in this study. As a prevalent vector of Dengue, Chikunguna, and Zika, further research on citronella oil's effectiveness as a larvicide against *A. aegypti* should be conducted.

The results from this study indicate that the essential oils of *C. nardus* are potential natural larvicides against *C. quinquefasciatus* and *C. nigripalpus* larvae. Further research is needed to examine the effects of citronella essential oil on organisms that live in the same aquatic breeding sites as mosquito larvae as well as the oil's effects on the abiotic factors such as the soil and water quality of the breeding sites.

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