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Impact of mycolactone produced by *Mycobacterium ulcerans* on life-history traits of *Aedes aegypti* (L.) and resulting habitat selection for oviposition

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Abstract. Buruli ulcer (BU) is a globally recognized, yet largely neglected tropical disease whose etiologic agent is *Mycobacterium ulcerans*. Although the exact mode of transmission is unclear, epidemiological evidence links BU incidence with slow-moving or stagnant, aquatic habitats, and laboratory-based experiments have shown disease manifestation in animals with dermal punctures. Therefore, hypotheses for transmission include contact with slow-moving aquatic habitats and associated biting aquatic insects, such as mosquitoes. Recent research demonstrated the toxin produced by *M. ulcerans*, mycolactone, is an attractant for adult mosquitoes seeking a blood-meal as well as oviposition sites. In the study presented here, we examined the impact of mycolactone at different concentrations on immature life-history traits of *Aedes aegypti*, which commonly occurs in the same environment as *M. ulcerans*. We determined percent egg hatch was not significantly different across treatments. However, concentration impacted the survivorship of larval mosquitoes to the adult stage ($p < 0.001$). Resulting adults also showed a slight preference, but not significant ($p > 0.05$), for oviposition in habitats contaminated with mycolactone suggesting a legacy effect.

INTRODUCTION

BURULI ULCER (BU) is a globally recognized, yet highly neglected tropical disease caused by *Mycobacterium ulcerans* (Merritt *et al*., 2010). Buruli ulcer is the third most common mycobacterial disease of humans after tuberculosis and leprosy (Vincent *et al*., 2014) mostly affecting individuals between the ages of 4 to 15 years old (Vincent *et al*., 2014; Williamson *et al*., 2008). The disease is prevalent in at least 33 countries, with most cases occurring in West Africa (Williamson *et al*., 2008; Merritt *et al*., 2010).

Mycobacterium ulcerans produces mycolactone, a cytotoxic and immunosuppressive polyketide-derived macrolide responsible for skin ulcerations, which are the primary BU disease manifestations (Mve-Obiang *et al*., 2003). The ulcers are painless; however, if not treated, they can expand and result in secondary infection, bone deformation, and osteomyelitis (Vincent *et al*., 2014). Though mortality is low, there is significant associated morbidity leading to socioeconomic burden and social stigma (Yeboah-Manu *et al*., 2013).

The exact mode of transmission of *M. ulcerans* is not known. Recent work using animal models has shown a dermal puncture is necessary to establish infection and BU disease pathology (Williamson *et al*., 2014). Epidemiological evidence links BU incidence with slow-moving aquatic habitats, especially in areas prone to anthropogenic disturbance and flooding. This evidence has been strengthened by the finding of

M. ulcerans DNA in cisterns and wells within villages (Merritt *et al*., 2010; Williamson *et al*., 2012, 2014; Wallace *et al*., 2017). Furthermore, insects, such as black flies (Diptera: Simuliidae), mosquitoes (Diptera: Culicidae) (Merritt *et al*., 2010), March flies (Diptera: Tabanidae), and sand flies (Diptera: Ceratopogonidae) (Quek *et al*., 2007), associated with one or the other of these environments (i.e., mosquitoes in ponds or cisterns) have been suggested as potential vectors. Moreover, *M. ulcerans* DNA has been identified in aquatic insects (Naucoridae and Belostomatidae) obtained from endemic areas in Africa (Williamson *et al*., 2008). In fact, under laboratory conditions, *M. ulcerans* was transmitted to mice by the bite of *M. ulcerans* infected aquatic hemipterans (Naucoridae) (Marsollier *et al*., 2002). Additionally, the mosquito *Aedes notoscriptus*, (Skuse) (Diptera: Culicidae) was able to mechanically produce infection in 2 of 11 mice whose tails had been dipped in *M. ulcerans* culture (Wallace *et al*., 2017).

A recent study in our laboratories demonstrated mycolactone influences behavior of the yellow fever mosquito, *Aedes aegypti aegypti*, (L.) (Diptera: Culicidae) (Sanders *et al*., 2017), which occurs in similar environments (Garnham *et al*., 1946; Christophers, 1960) as *M. ulcerans* (Williamson *et al*., 2012). In the study, 29% more were attracted to blood-feeders treated with 1.0 µg/ml mycolactone compared to control feeders. They further demonstrated naïve mosquitoes (never exposed to *M. ulcerans* or mycolactone) preferred to oviposit (64%) in areas containing mycolactone at the same concentration (1.0 µg/ml) that enhanced attraction to the blood-feeders (Sanders *et al*., 2017). However, the impact of mycolactone on subsequent mosquito development and resulting adult behavior is not known.

Since many mosquitoes (e.g., *Ae. aegypti*) have strong oviposition preferences for sites with bacteria (Ponnusamy *et al*., 2008), the relationship between BU and *Ae. aegypti* may become even more complex if *Ae. aegypti* prefer to oviposit in sites similar to their larval habitat. In fact, volatile organic compounds released by bacteria have been demonstrated to drive mosquito preferences to oviposit in bacteria-present vs. bacteria-absent sites (Corbet, 1985; Albeny-Simoes *et al*., 2014). A potential source of these volatiles is the by-products produced by bacterial quorum sensing (QS) or other secondary metabolism, which are modulators of inter-kingdom behavior, especially insects (Tomberlin *et al*., 2016).

Quorum sensing is a process coordinating gene expression according to the density of a bacterial population (Miller & Bassler, 2001). This process allows for population-level physiological and other phenotypical shifts by bacteria, such as biofilm formation or virulence expression (Miller & Bassler, 2001). A recent laboratory study demonstrated QS by the human dermal commensal, *Staphylococcus epidermidis* enhanced mosquito attraction to a blood-feeders by 74% versus the strain unable to QS (Zhang *et al*., 2015).

The macrolide structure of mycolactone suggests the possibility that the molecule may be an antagonist to bacteria with QS machinery (similar to acyl-homoserine lactones) or may serve as a regulator of secondary metabolism (Romero *et al*., 2011). And, as previously stated, mycolactone is a known mosquito attractant (Sanders *et al*., 2016). Based on these and previously published data, we sought in the current study to determine whether: 1) immature *Ae. aegypti* development and survivorship in the presence of mycolactone were dose-dependent, and if so, 2) do resulting *Ae. aegypti* adults prefer to oviposit in habitats containing mycloactone at rates similar to those experienced during larval development.

MATERIALS AND METHODS

Insect Colonies

Aedes aegypti (Liverpool strain) was used in this study due to the common occurrence of this species in areas endemic to *M. ulcerans* as mentioned in the introduction. *Aedes aegypti* was maintained in a colony housed in a walk-in growth chamber at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH at the Forensic Laboratory for Investigative Entomological

Sciences (FLIES) Facility (Texas A&M University, College Station, TX, USA). Eggs were placed in 1 L of distilled water held in containers (17.5 cm × 12 cm × 4.5 cm) at room temperature. Resulting larvae were separated into similar containers at a density of 100-200 larvae/L. Larvae were provided fish food *ad libitum* (TetraMin diet by Tetra Blacksburg, VA, USA) to avoid overfeeding. Distilled water was added to the containers as needed; containers were checked every 12 h for the presence of pupae, which were then partitioned into 60 ml cups (containing 40 ml of distilled water) at a density of 50 pupae/cup. These cups were placed individually inside an insect cage (30 × 30 × 30 cm), and pupae were monitored for adult emergence. Newly emerged adults were provided with a 10% sucrose solution via a wet cotton ball placed on top of the cage.

Mycolactone

Mycolactone at 1.0 µg/ml, 0.5 µg/ml, and 0.05 µg/ml concentrations were used in this study. Mycolactone was prepared using previously described methods (Adusumilli *et al.*, 2005) with slight modifications; specifically, *M. ulcerans* Agy99 was grown on M7H10 plates. Bacteria were scraped from the plates, dried and weighed. Mycolactone was isolated from acetone soluble lipids (ASLs) using centripetal chromatography by running concentrated lipids (dried down ASLs resuspended in 2 ml 96:4 dichloromethane: MeOH) through a chromatotron to separate individual lipid species using 96:4 dichloromethane: MeOH followed by 90:10 chloroform: MeOH with fraction collection and visualization of the UV-active species and further fractionation and collection. Fractions were analyzed by TLC ran in 90:10:1 chloroform:methanol:water solvent using a mycolactone control and visualized with ceric sulfate-ammonium molybdate in 2M sulfuric acid stain with Rf values compared against a mycolactone control. A cytopathicity assay was conducted to confirm activity as previously described (Mve-Obiang *et al.*, 2003). Mycolactone concentration was extrapolated from *M. ulcerans* cell weight and corresponding colony count, where one cell was estimated to produce approximately 1 pg of mycolactone. This information was used along with the qPCR values of *M. ulcerans* from environmental samples to choose mycolactone concentrations used in this study, with an effort to represent a range of detected concentrations corresponding to qPCR-assayed environmental matrices collected from Ghana and Benin (Williamson *et al.*, 2012) [HRJ, unpublished data]. Prepared mycolactone was solubilized with 95% ethanol and stored in amber vials placed in the dark at room temperature to prevent degradation due to ultraviolet light (Marion *et al.*, 2012).

Larval Growth/Survivorship Assay

All experiments were conducted under the laboratory conditions previously described (Sanders *et al.*, 2017). For the larval growth/survivorship assay, 40 *Ae. aegypti* eggs total, taken from multiple females from the colony, were placed in a round glass jar (236 ml) (Packaging Options Direct, Louis, MO, USA) containing 55 ml of distilled water. This density was selected based on preliminary experiments where the greatest level of survivorship to the adult stage was determined. Approximately 30-50 mg Tetramin fish food was placed in the water at the time the eggs were introduced. Larvae were reared as previously described.

For the treatments, 50 µl mycolactone was applied using micropipette (Eppendorf, NY, USA) at 1.0 µg/ml (high), 0.5 µg/ml (medium), and 0.05 µg/ml (low) concentration with 95% ethanol serving as the solvent. Ethanol (50 µl) alone served as the positive control; a negative control (nothing applied) was also used. Jars with three concentrations (1.0 µg/ml, 0.5 µg/ml and 0.05 µg/ml) were used as the containers for each replicate during the experiment. Each mycolactone treatment was added to a glass jar containing distilled water (55 ml) and allowed to ventilate for 30 min prior to the introduction of mosquito eggs. Glass jars (replicates) were then placed individually in a mosquito-breeder (L: 21cm × W: 12 cm) (BioQuip, CA, USA). To avoid sampling bias, replicates were checked every 12 h for pupae and adult emergence (total percent recorded). The sex of each emerged adult was

also recorded, and the percentage hatch rate and larval survivorship to adulthood were measured. Four trials of the experiment were completed.

Oviposition assay

Based on results from the larval growth/survivorship assay, mosquitoes were reared in the presence of mycolactone at 0.5 µg/ml ethanol as it yielded the most similar adult emergence patterns to those seen in controls when compared to the other treatments. The quantity of mycolactone/solvent (50 µl) applied was adjusted for the volume of water within a container. Cluster eggs were placed in 1 L of distilled water treated with either 0.5 µg mycolactone/ml ethanol, just ethanol or a negative control (nothing applied) as control, and held in 30 cm × 23 cm × 5 cm mosquito pans (Bioquip, CA, USA) in the walk-in incubator at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH. Larvae were reared and monitored as described previously until emergence. Adult mosquitoes aged 5-10-d-old were then starved for 24 h prior to being provided a blood-meal using methods previously described (Sanford & Tomberlin, 2011).

Approximately 72 h after blood-feeding, three, 150 ml black containers were placed equidistant from one another in triangulated fashion in each cage representing each treatment. In each container, a single filter paper (11 cm in diameter, Whatman No. 1) was provided as an oviposition site as a normal substrate for eggs collection (Imam *et al.*, 2014). Each filter paper, which represented a treatment or control (0.5 µg mycolactone, ethanol, blank) was divided equally, where half of the paper was treated, and the paper was placed in water with the orientation of the treated side of the filter paper-oriented randomly east or west in the cage. *Ae. aegypti* were allowed to oviposit for 72 h, after which, the filter papers were removed, and dried in the incubator for four days. Eggs present on each half were then tabulated. Three trials of the experiment were completed. For each trial, the location of the treatment within the triangulated positions was rotated as well as the orientation of the treatment east or west.

Statistical Analysis

An analysis of variance (ANOVA) was used to assess larval growth/survivorship assay data (JMP® Pro 12.0.1, Cary, NC, USA). Tukey's multiple comparison procedure and statistical test were used to separate means following a significant F test. The alpha value was set at $p < 0.05$. For the oviposition study, an ANOVA was used to determine differences in the number of eggs deposited on the three treatments present in a given cage. For eggs deposited on half of the filter paper with either mycolactone or ethanol, the probability (P) of response (i.e., oviposition) by *Ae. aegypti* was examined for a significant difference ($p < 0.05$). Furthermore, a comparison of mosquito responses across doses was conducted with PROC GLIMMIX (SAS University Edition, Version 9.4), a generalized linear mixed model (GLMM). The odds of treatment/blank control (i.e., attraction and oviposition) by *Ae. aegypti* to the different treatments was examined for significant difference ($p < 0.05$) between mycolactone and ethanol treatments. Replicate was included in the model as a random factor.

RESULTS

Larval Hatch/Survivorship Assay

No significant ($F = 0.781$; $df = 4, 59$; $p = 0.544$) difference in egg hatch was determined across treatments. Furthermore, no significant ($F = 0.738$; $df = 12, 59$; $p = 0.707$) interaction was determined between trial and treatment. Although no difference in response of mosquitoes to treatments was detected (i.e. treatment by trial interaction), a trial effect was determined ($F = 9.099$; $df = 3, 59$; $p < 0.001$). However, it should be noted the order of treatment responses was consistent across trials. Trials 1 and 2 were significantly ($p < 0.05$) different from trials 3 and 4. The average egg hatch in trial one and two was 97.50% ± 0.01% (Figure 1a) and 80.10% ± 0.03% (Figure 1b) in trials three and four.

Significant difference ($F = 10.085$; $df = 4, 53$; $p < 0.001$) across treatments was determined for survivorship from egg to pupal and adult stage. No significant ($F = 1.070$; $df = 12,$

53; p = 0.414) interaction was determined between trial and treatment. No trial effect was determined (F = 1.436; df = 3, 53; p = 0.249). The average survival of eggs to the pupal and adult stage for the controls was 81% or greater. Survival of those exposed to the different mycolactone treatments was greatest for the middle dose (0.5 µg/ml; 58%), which was not significantly different from the controls; however, survival, when exposed to this treatment, was almost double of what was observed for those assigned the high (1.0 µg/ml) and low (0.05 µg/ml) dose treatments (~30–39% respectively) (Figure 2). No mortality was observed at the pupal stage.

Significant differences (F = 4.837; df = 4, 52; p = 0.004) in development time to the pupal stage were determined across treatments. No significant interaction (F = 1.711; df = 12, 53; p = 0.110) was determined

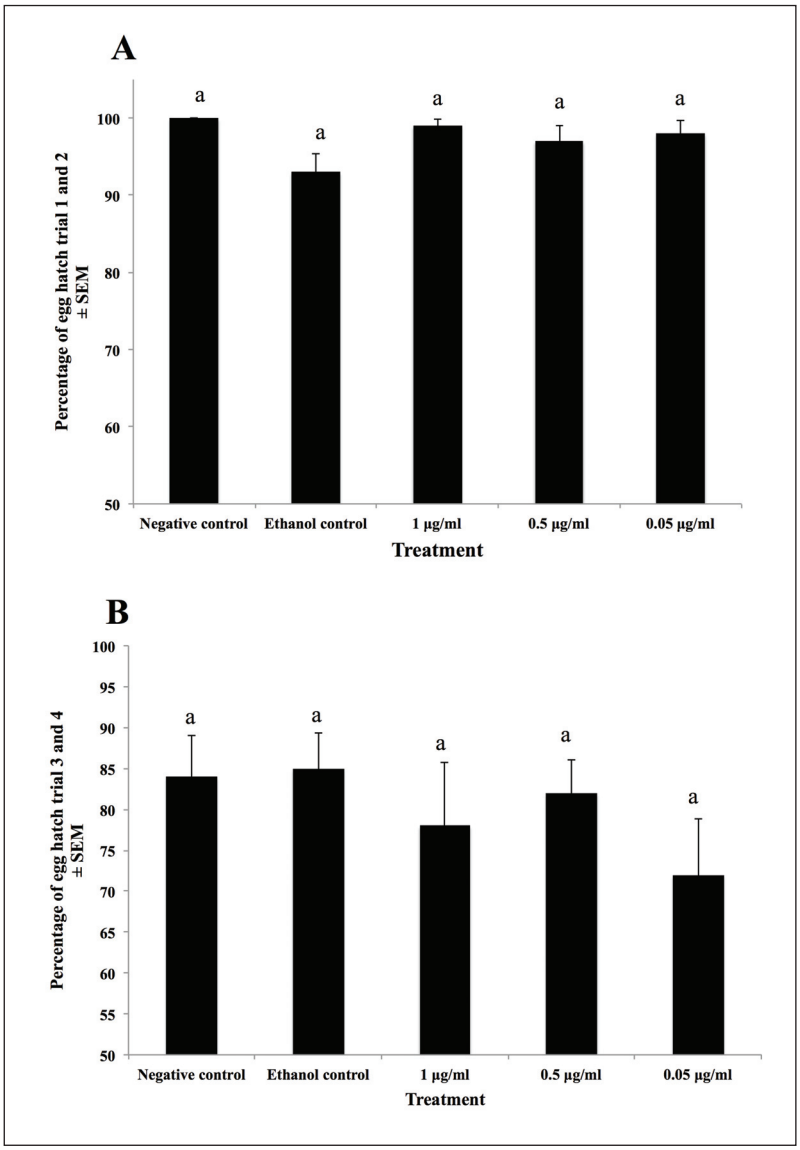

Figure 1. Percentage of *Ae. aegypti* egg hatch (n = 4) ± SEM exposed to three concentrations of mycolactone, negative control, or ethanol control at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH for (A) trials 1 and 2 for (B) trials 3 and 4 combined.

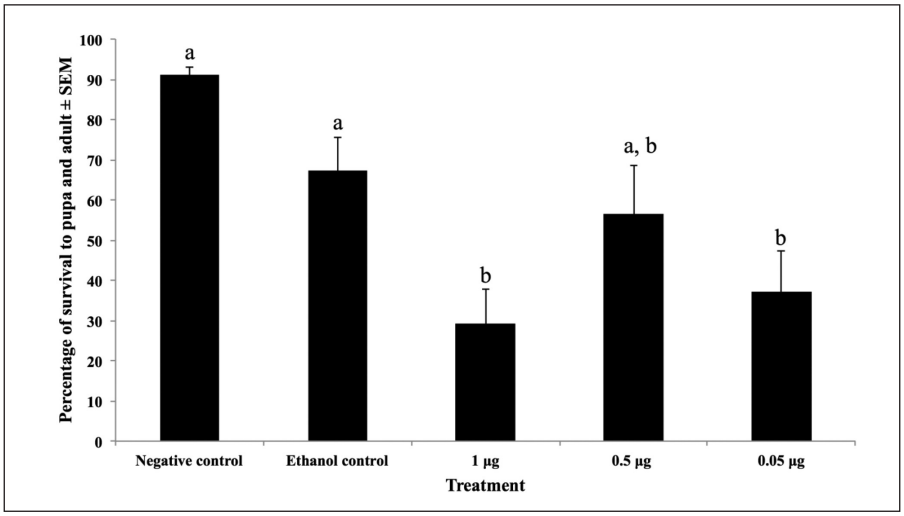

Figure 2. Percentage of *Ae. aegypti* survival from egg to pupal and adult stages (n = 4) ± SEM exposed to three concentrations of mycolactone, negative control, or ethanol control at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH.

Table 1. Mean ± SEM of time (d) from egg to pupae and adult of *Ae. aegypti* exposed to different concentration of mycolactone, as well as negative control and ethanol control at 25.0° ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH

Mean time from egg to pupae (d) Mean ± SEM		
Treatment	Trial 1, 3 and 4	Trial 2, 3 and 4
Negative control*	7.35 ± 0.15[b]	7.57 ± 0.06[a]
Ethanol control	6.87 ± 0.20[b]	7.57 ± 0.41[a]
1 µg/ml	8.07 ± 0.46[a]	8.33 ± 0.40[a]
0.5 µg/ml	7.33 ± 0.18[b]	7.58 ± 0.10[a]
0.05 µg/ml	7.48 ± 0.29[a,b]	7.63 ± 0.30[a]

Mean time from egg to adult (d) Mean ± SEM			
Treatment	Trial 1	Trial 3	Trial 2 and 4
Negative control*	9.53 ± 0.09[a]	13.70 ± 0.06[a]	12.77 ± 0.05[a]
Ethanol control	9.55 ± 0.07[a]	13.80 ± 0.00[a]	12.63 ± 0.08[a]
1 µg/ml	9.33 ± 0.00[a,b]	13.30 ± 0.30[a]	12.48 ± 0.11[a]
0.5 µg/ml	9.24 ± 0.24[a,b]	13.80 ± 0.00[a]	12.55 ± 0.13[a]
0.05 µg/ml	9.00 ± 0.00[b]	13.80 ± 0.35[a]	12.50 ± 0.13[a]

*Negative control was no treatment (just water).

between trial and treatment. However, a trial effect was determined ($F = 3.750$; df = 3, 53; $p = 0.020$). Therefore, the results for development time from egg to the pupal stage were grouped by trial. The average time from egg to pupa ± SEM is shown in (Table 1).

For trials 1, 3 and 4, significant ($F = 6.129$; df = 4, 37; $p = 0.002$) the difference in development time from egg to the pupal stage was determined across treatments. From these, individuals in the negative control group needed 7.35 d or less to pupate. The development time for those exposed to the high dose for trials 1,3, and 4 was significantly greater (~8 d) than for individuals in other treatments (~7 d for the mean of control, low and middle doses). Furthermore, a significant ($F = 2.796$; df = 8, 37; $p = 0.026$) interaction

was determined between trial and treatment. For trials 2, 3 and 4, treatment did not significantly (F = 1.820; df = 4, 40; p = 0.155) impact development time from egg to the pupal stage.

Significant difference (F = 5.332; df = 4, 52; p = 0.002) in development time to the adult stage was determined across treatments (Table 1). No significant (F = 0.844; df = 12, 52; p = 0.607) interaction was determined between trial and treatment. A trial effect was determined (F = 760.306; df = 3, 53; $p < 0.001$) in development time to the adult.

A significant (F = 5.586; df = 4, 11; p = 0.024) difference in development time from egg to the adult stage was determined across treatments in trial 1. Individuals in the ethanol control groups needed a maximum of 9.55 d to become adults, which was greater than observed for the treatments. The development time for those exposed to the high dose (1 µg/ml) was significantly greater (~9.33 d) than for individuals in other treatments (~9.12 d for mean of ethanol control, low and middle doses). Trial 1 was significantly ($p < 0.05$) different from trials 2, 3 and 4, but none of these trials demonstrated a significant (trials 2 and 4: F = 1.486; df = 4, 29; p = 0.244; trial 3: F = 1.910; df = 4, 10; p = 0.228) impact on development from egg to adult.

Oviposition Assay

Mosquitoes were reared in containers treated with 0.5 µg/ml mycolactone or reared in the absence of mycolactone (ethanol as control); the number of eggs deposited in sites with the same mycolactone dose or the controls was determined (Figure 3). Mosquitoes reared in the control did not show a significant ($p > 0.05$) preference for such sites when ovipositing. However, mosquitoes reared in the presence of mycolactone were slightly more likely to deposit eggs on the portion of filter paper treated with either mycolactone ($p < 0.0001$, X^2 = 184.81, log odds of responding to mycolactone versus the blank = 0.69 ± 0.05, Figure 4) or ethanol ($p < 0.0001$, X^2 = 15.65, log odds of responding to ethanol versus the blank = 0.23 ± 0.06, Figure 3) relative to the untreated half. In contrast, mosquitoes initially reared in the presence of ethanol appeared to be repelled by the presence of either mycolactone ($p <$ 0.0001, X^2 = 28.97, log odds of responding to

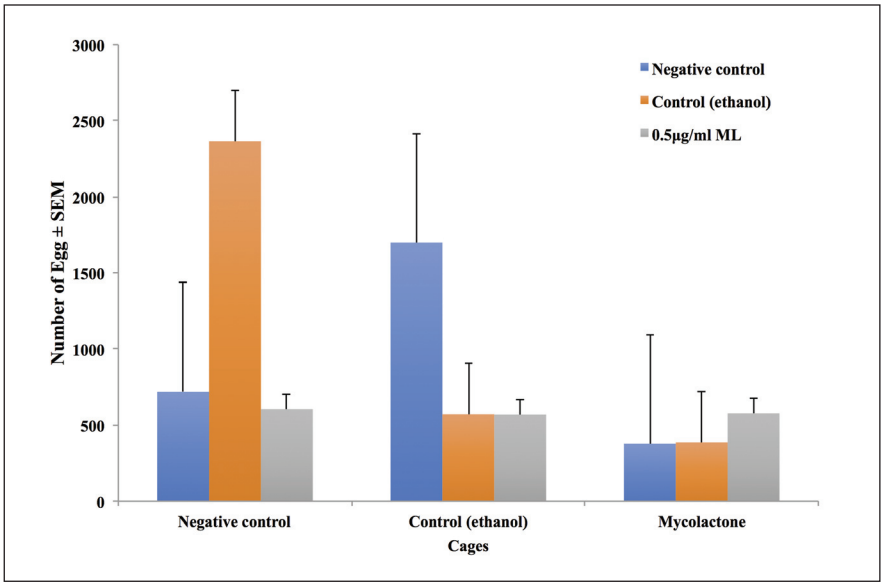

Figure 3. Number of *Ae. aegypti* eggs laid on filter paper treated with ethanol or mycolactone by adults reared in the presence of 0.5 mycolactone µg/1 ml ethanol, 1 ml ethanol and negative control at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH during larval development.

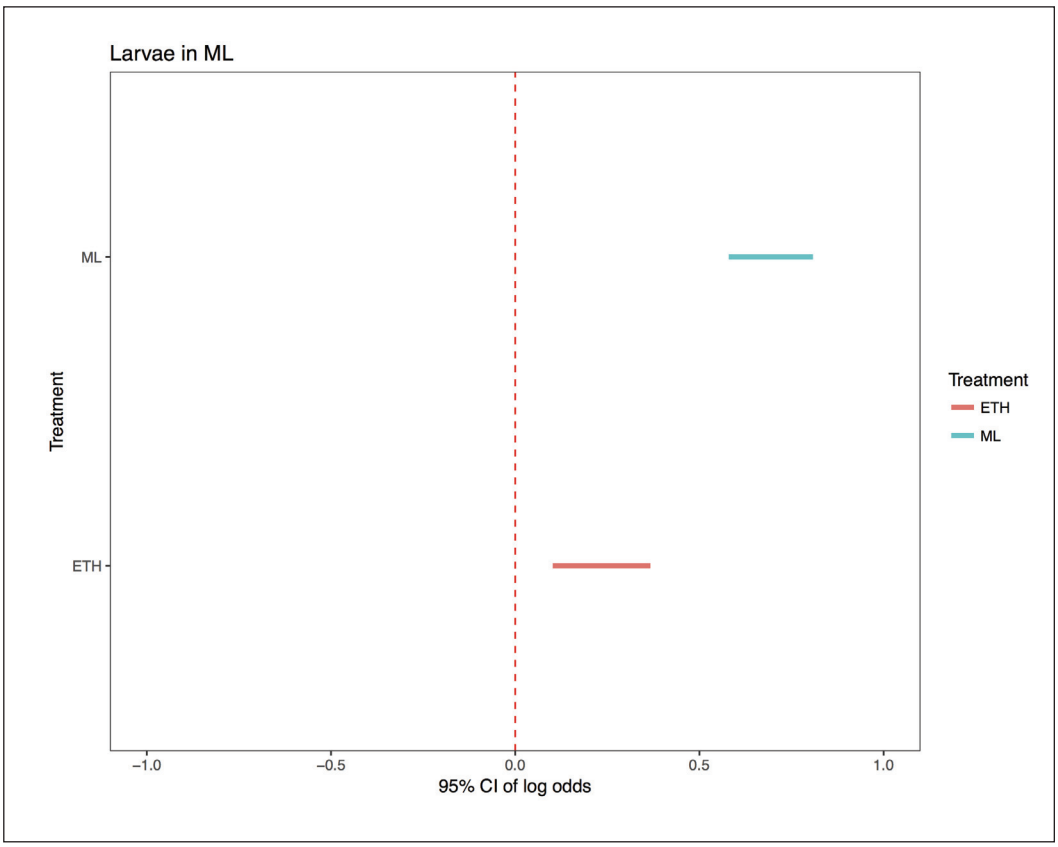

Figure 4. Log odds of *Ae. aegypti* laying eggs on filter paper treated with 0.5 µg mycolactone at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH when reared in water containing 0.5 µg mycolactone/1 ml ethanol. *ETH= Ethanol; ML= Mycolactone.

mycolactone versus the blank = -0.26 ± 0.05, Figure 5) or ethanol ($p < 0.0001$ $X^2 = 29.90$, log odds of responding to ethanol versus the blank = -0.27 ± 0.05, Figure 5).

DISCUSSION

The work presented here revealed mycolactone impacts larval development and survivorship of *Ae. aegypti*. Mycolactone impacted mosquito development from the egg to the pupal stage. Immature mosquitoes in the control groups needed approximately 7.2 d to reach the pupal stage, which is similar to results from a past study conducted under similar conditions (Christophers, 1960; Imam *et al.*, 2014). However, those exposed to the high mycolactone dose needed approximately 15% more time to reach the pupal stage. Similarly, development from egg to adult exhibited a treatment effect (Table 1); however, this difference was never more than half a day and was most likely due to scheduled observations (e.g., observations every 12 h) rather than biological significance. Additional studies with more refined observation periods should be conducted to determine whether the response is statistically different. Furthermore, fieldwork should be conducted to determine whether these data translate to natural populations.

In addition, the response trended by dose where the high and low doses reduced survivorship from egg to pupa and adult (Figure 2), and the middle dose appeared to be optimal. The survival of eggs to the pupal stage in the control groups was above 80%, while those exposed to the high and low mycolactone dose were between 35 and 40%.

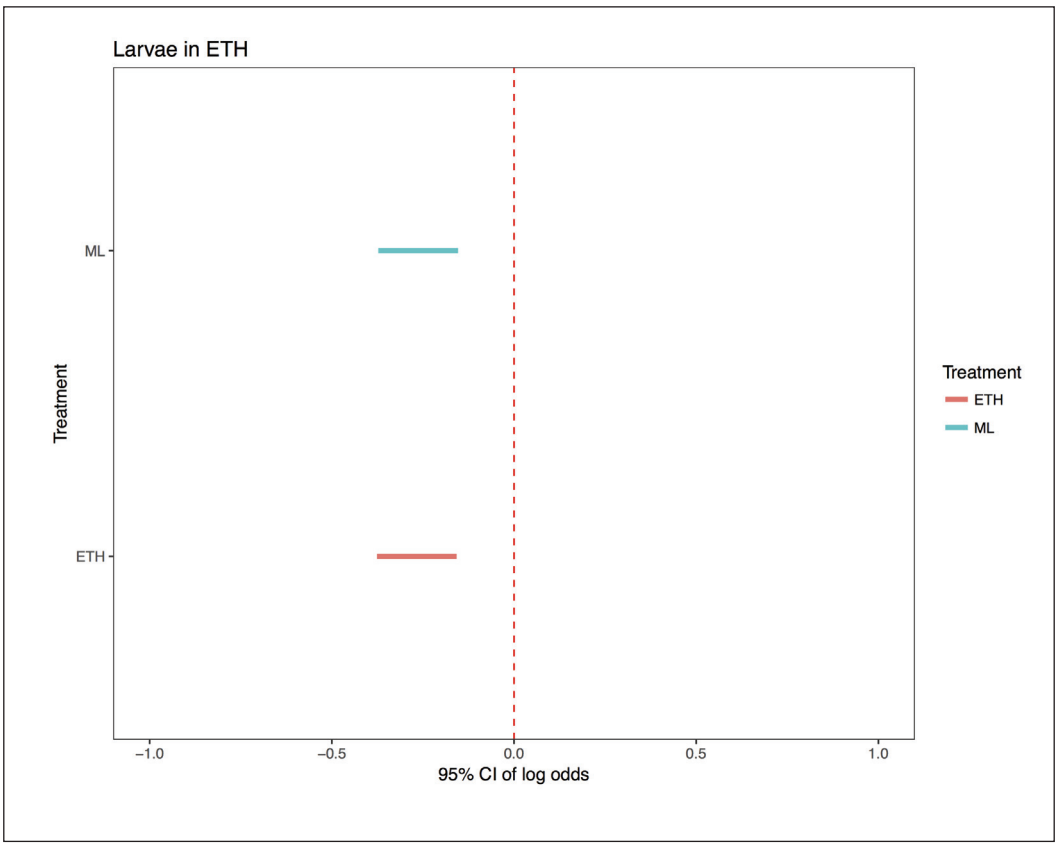

Figure 5. Log odds of *Ae. aegypti* laying eggs on filter paper containing 0.5 µg mycolactone/1 ml ethanol at 25.0°C ± 2.5°C, 12:12 L:D, and 70.0% ± 5.0% RH when reared in water containing 1 ml ethanol. *ETH= Ethanol; ML= Mycolactone.

In contrast, survivorship of eggs to the pupal stage from those exposed to the middle dose, while still lower than the control groups, had a 58% survivorship.

Throughout the course of these experiments, immature mosquitoes exposed to the middle mycolactone dose consistently produced greater survivor rates compared to other treatments, and yielded survivorship closest to the control group, possibly indicating a concentration window of suitability. These results are not surprising as such optima in mosquitoes have been recorded in response to a number of abiotic conditions. For example, too high temperatures can result in larval mortality, while too low temperatures stalled development (Couret *et al.*, 2014). Specifically, room temperatures above 30°C resulted in larval mortality, while temperatures between 20–30°C resulted in optimal development and survivorship.

While not examined in this study, one explanation for the specific dose-response to mycolactone on mosquito larval development could relate to shifts in available nutrients. As previously indicated, *M. ulcerans* occurs in mostly lentic habitats where *Ae. aegypti* larvae also occur (Wallace *et al.*, 2010; McIntosh *et al.*, 2014). Bacteria in these habitats are known to breakdown organic matter, which then serves as a primary food substrate of the mosquito larvae (Kaufman *et al.*, 1999). Recent research published by Souza *et al.* (2019) demonstrated shifts in microbial communities impact *Ae. aegypti* development time; more specifically, pupal development can be delayed significantly depending on which microbes were present during larval

development (ranging from 7.5 d for the control to 18.4 d when larvae were presented with *Escherichia coli* (Souza *et al*., 2019).

The macrolide structure of mycolactone is similar to some QS molecules and has been hypothesized to function as a modulator of QS machinery or toward other mechanisms of secondary metabolism. Additionally, our group has found that mycolactone is a QS antagonist to some commensal and environmental bacteria. In the case of the present study, mycolactone present in the growth environments at certain concentrations could be inhibiting other microbes (i.e., bacteria) competing with *M. ulcerans* for similar resources. This inhibition could, in turn, be suppressing bacterial populations within the growth medium that are directly necessary for optimal larval mosquito development. Work is currently underway by our group for further examination with respect to *M. ulcerans*, and corresponding mycolactone, interactions with mosquitoes under natural, polymicrobial conditions.

Another possibility is that mycolactone could be impacting mosquito gut bacteria, which also play a crucial role in mosquito development. While not with our species, Chouaia *et al*. (2012) determined that *Asaia* symbiotic bacteria are beneficial in the development of immature *Anopheles stephensi*, (L.) (Diptera: Culicidae). Development of *An. stephensi* larvae reared in a habitat with rifampicin were delayed two to four days compared with those in the control (Chouaia *et al.*, 2012). In the current study, mycolactone could have a similar impact; however, the associated bacterial community was not measured in this study. Future studies examining the effects of *M. ulcerans* and mycolactone on the microbial community associated with *A. aegypti* will shed critical light on our understanding of the mechanisms linking *M. ulcerans* and the yellow fever mosquito.

With regards to the behavioral study, our data indicate mosquitoes reared in the presence of mycolactone could be biased with regards to oviposition site selection. In this case, mosquitoes were more likely to deposit eggs in an environment containing mycolactone if they themselves were reared in such an environment. McCall and Eaton (2001) demonstrated adult *Culex quinquefasciatus*, Say (Diptera: Culicidae) reared in the presence of skatole (innately a repellent at high concentrations) or *p*-Cresole were more likely to deposit eggs in environments containing this compound. Accordingly, Sanford *et al.* (2012) determined similar "learning" abilities with adults being able to recognize and respond to these odors in anticipation of securing a food source, thus lending support the legacy effect hypothesis. In this case, volatile compounds potentially associated with microbes in the aquatic environment elicit the mosquito response. Others have demonstrated similar mosquito responses. For example, *Ae. aegypti* and *Anopheles gambiae*, Giles (Diptera: Culicidae) utilize indole (i.e., microbial by-product) as a means for determining oviposition sites (Bohbot *et al*., 2011). Finally, as mentioned previously, gravid *Ae. aegypti* use volatiles in the form of carboxylic acids and methyl esters emitted from alpha and gamma proteobacteria as potent oviposition stimulants (Ponnusamy *et al*., 2008).

But why would mosquitoes in the current study deposit eggs in an environment not conducive for optimal larval development and survivorship? One explanation could be due to the mosquito population used in the current study are from a colony maintained without previous selective pressures by *M. ulcerans*, which could explain the partial morality we observed. Much like mosquitoes developing resistance or tolerance to insecticides, mosquitoes breeding in natural habitats with *M. ulcerans* could be undergoing the same, resulting in resident populations highly responsive to mycolactone, to locate hosts or oviposition sites.

A competing hypothesis is that the interaction between *M. ulcerans* and mosquitoes could be microbial manipulation of its host, whereby the microbe receives an advantage (i.e., distribution as previously discussed) and not the host. Such behavioral shifts are well documented for other microbes or parasites. For example, crickets infected with a parasite seek out aquatic habitats which result in their mortality but survivorship of the parasite (Hughes *et al*., 2012).

Similarly, ants infected with a fungus exhibit similar behaviors resulting in enhance dispersal of the pathogen but to the detriment of the host (Hughes, 2013). Such a fascinating question beckons greater examination in future research as resulting data could provide insights into the evolutionary relationship between *M. ulcerans*, its toxin, and mosquito behavior. A potential scenario could be regions endemic with *Ae. aegypti* and *M. ulcerans* result in adult mosquitoes serving as a mechanism for distributing of the pathogen to new locations through subsequent oviposition events.

Results from the current study demonstrate a potential ecological link between *M. ulcerans* and *Ae. aegypti* in endemic environments where both species occur as well as other pathogens including yellow fever and dengue. Understanding this could prove crucial for deciphering the etiology of the pathogen and its mode of transmission. However, additional research is needed with viable *M. ulcerans* cells to determine whether mycolactone production, the production of other secondary metabolites or compounds, may impact mosquito development and oviposition site selection. If these results remain true, a synergism between the incidence of BU and yellow fever in non-immunized individuals could be possible and would require further investigation in these endemic areas. Furthermore, while we examined three concentrations of mycolactone encompassing concentrations detected in nature, future studies should explore multiple concentrations at a finer scale to determine the specific range eliciting the physiological and behavioral responses observed in the research presented in this manuscript. Additionally, determining the impact of mycolactone on other life-history traits (i.e., development over time) and morphometrics (e.g., size of adults) of mosquitoes is needed to better understand these interactions. Furthermore, while we verified UV- and biological activity, and retention factor (Rf) against a mycolactone control, it cannot be ruled out that minor mycolactone congeners or other minor lipids that ran within the same Rf value were also constituents of the purified mycolactone used in these experiments and might have affected mosquito development and behavior.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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