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Mycobacterium ulcerans toxin, mycolactone may enhance host-seeking and oviposition behaviour by *Aedes aegypti* (L.) (Diptera: Culicidae)

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Summary

The ecological functions of many toxins continue to remain unknown for those produced by environmental pathogens. *Mycobacterium ulcerans*, the causative agent of the neglected tropical disease, Buruli ulcer, produces a cytotoxic macrolide, mycolactone, whose function(s) in the environment remains elusive. Through a series of dual-choice behaviour assays, they show that mycolactone may be an interkingdom cue for the yellow fever mosquito, *Aedes aegypti*, seeking blood-meals as well as oviposition sites. Results provide novel insight into the evolution between bacteria and potential vectors. While further studies are needed to determine if mycolactone is an actual signal rather than simply a cue, this discovery could serve as a model for determining roles for toxins produced by other environmental pathogens and provide opportunities for developing novel strategies for disease prevention. The relationship between *M. ulcerans*, mycolactone, and *Ae. aegypti* further suggests there could be an amplification effect for the spread of pathogens responsible for other diseases, such as yellow fever and dengue.

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Introduction

Environmental pathogens such as *Mycobacterium ulcerans* (Merritt *et al.*, 2010; Yotsu *et al.*, 2015), *Legionella pneumophila* (Whiley *et al.*, 2014; Falkinham, 2015; Falkinham *et al.*, 2015), *Vibrio cholera* (Vezzulli *et al.*, 2010; Lutz *et al.*, 2013), and many others spend a substantial part of their lifecycle outside human hosts and only cause disease when transmitted from direct contact with contaminated food, air, water, soil, living reservoirs or vectors (Walsh *et al.*, 2011). As with all microorganisms, environmental pathogens must maintain a wide range of gene products and secondary metabolites for access to nutrient sources and adaptation to the broad array of stresses encountered for survival in the soil, water, air or other natural environments. Many of these gene products are necessary for growth and development; however, many secondary metabolites are utilized for survival in the environment against other prokaryotic or eukaryotic organisms (Demain and Fang, 2000; Hibbing *et al.*, 2010; Pierson and Pierson, 2010; Ghoul and Mitri, 2016). These secondary metabolites may thus serve an environmental purpose to the pathogen, while also increasing the ability to cause disease once in a host (Demain and Fang, 2000; Hibbing *et al.*, 2010; Pierson and Pierson, 2010).

For many environmental pathogens, data regarding modulation of secondary metabolite production, and the role these small molecules serve for a pathogen in their natural environments are lacking. For example, the environmental pathogen *M. ulcerans* (MU) secretes an immunosuppressive polyketide-derived lipid called mycolactone that is a major virulence determinant in human hosts (George *et al.*, 2000). Mycolactone is an exotoxin that destroys cells in the host epidermis, releasing lipids, which allows it to cause pathology extending further than the site of bacterial colonization (George *et al.*, 2000; Johnson *et al.*, 2005). This mechanism, and resulting pathology, is a hallmark for the neglected tropical disease, Buruli ulcer (BU) (Sarfo *et al.*, 2016). The effects of mycolactone on eukaryotic cells are well documented since this is essential for disease manifestation, but like other secondary metabolites, mycolactone most likely plays an important role in the population biology and community

ecology of the species while in its natural environmental niche (Marsollier *et al*., 2007). However, though genomic findings suggest mycolactone has been an evolutionarily important molecule to MU (Stinear *et al*., 2005, 2007), its hypothesized role(s) in MU survival and persistence in the natural environment is unknown.

MU, and also BU disease prevalence, are associated with slow moving, aquatic environments where MU DNA has been found associated with invertebrates, water, and biofilms (Kotlowski *et al*., 2004; Mosi *et al*., 2008; Williamson *et al*., 2008; Merritt *et al*., 2010; Williamson *et al*., 2012; Willson *et al*., 2013). Despite this, the mode of transmission to human hosts remains a mystery in the field. Two recent hypotheses for MU transmission are: MU transmission occurs through a puncture wound into MU-contaminated skin following exposure to a MU contaminated environment; and, receiving less evidence, inoculation by an insect vector (Portaels *et al*., 1999; Marsollier *et al*., 2002; Merritt *et al*., 2005; Williamson *et al*., 2012; 2014; Garchitorena *et al*., 2015).

The second hypothesis is supported through early epidemiological and field data correlating BU risk with mosquitoes (Diptera: Culicidae) (Johnson *et al*., 2007; Quek *et al*., 2007; Lavender *et al*., 2011). Mosquito larvae are associated with habitats that consist most often of lentic water and thus share an environment consistent with MU (Clements, 1999). Additionally, mosquito larvae have a labral head fan that is used as a filtration method for filtering particulate within the water column, including microbes. The feeding method of mosquito larvae allows the possible consumption and concentration of MU (Wallace *et al*., 2010).

Studies have examined the role of mosquitoes as vectors of MU, as well as the role mosquitoes may have in the movement of the pathogen through an aquatic food web (Wallace *et al*., 2010). Results from Wallace *et al*. (2010) showed high MU concentrations were present for up to six days in the mosquito larval gut and demonstrated that MU could be transmitted through different trophic levels of the aquatic food web. *M. ulcerans* was not detectable in the adults, suggesting that mosquitoes are unlikely serving as a biological vector. However, MU was detected on external appendages of some of the tested mosquitoes suggesting the possibility for a role in mechanical transmission (Wallace *et al*., 2010). Therefore, questions remain as to whether an arthropod could mechanically transmit MU (either from inoculation by a MU contaminated mosquito or introduced via puncture of MU contaminated skin by mosquito attempting to feed), and if the MU exotoxin mycolactone serves as an attractant for potential vectors.

Recent studies have shown that some microbial metabolites (e.g., quorum sensing [QS] molecules) influence insect behaviour (Ma *et al*., 2012; Tomberlin *et al*., 2012; Zhang *et al*., 2015; Liu *et al*., 2016), suggesting that there may be widespread interkingdom interactions between insects and microbes that have biological and ecological importance. Other studies have shown that the QS molecule indole produced by *Escherichia coli* and other bacteria (Lee *et al*., 2007) is a known fly attractant (Ashworth and Wall, 1994). Additionally, we showed that volatiles produced by single and mixed-species of bacteria had behaviour fitness effects on black soldier flies, *Hermetia illucens* (L.), (Diptera: Stratiomyidae) (Zheng *et al*., 2013). Quorum sensing compounds allow exchange of chemical signals for bacteria to monitor their population density, as well as to regulate gene expression in a population-dependent manner (Lowery *et al*., 2008). These compounds provide a cell-to-cell communication pathway in bacteria (Waters and Bassler, 2005) and have been used to regulate interspecies and interkingdom interactions (Lowery *et al*., 2008). Furthermore, QS and interkingdom interactions has been observed in interactions between bacteria and mosquitoes (Lowery *et al*., 2008; Ezenwa *et al*., 2012; Ezenwa and Williams, 2014), where mosquitoes 'eavesdrop' on the communication of bacteria, that subsequently influence mosquito decision-making and behavioural responses (Ponnusamy *et al*., 2010; Verhulst *et al*., 2010a). The macrolide structure of mycolactone suggests the possibility that the molecule may be an antagonist to bacteria with quorum sensing machinery (similar to acyl homoserine lactones) or may serve as a regulator of secondary metabolism (Hashimoto *et al*., 2011; Romero *et al*., 2011). However, QS compounds produced by MU and their possible influence on arthropod behaviour, particularly mosquito attraction and oviposition, are unknown.

Therefore, to better understand the biological role of mycolactone, interkingdom interactions between MU and mosquitoes, and the ecological implications in terms of disease prevalence and pathogen dispersal, the following was addressed: (1) the behavioural response of adult yellow fever mosquitoes, *Aedes aegypti aegypti*, (Linnaeus *in* Hasselquist) (Diptera: Culicidae), which commonly occurs in areas endemic to MU, to mycolactone with regard to host-seeking behaviour, and (2) oviposition site selection.

Results

Mosquito response to blood-feeders treated with mycolactone

The percent attraction response of *Ae. aegypti* adults to blood-feeders treated with mycolactone at different concentrations in comparison to a control are presented in Tables 1–3. Mosquito responses at the low dose (0.05 μg ml^{-1}) were not significant. Accordingly, no significant difference in mosquito attraction to the treatment or control was determined between trials ($G_H = 5.895$, $df = 3$, $p = 0.117$) (Table 1).

Mosquitoes tested with the intermediate (0.50 μg ml^{-1}) dose were attracted to the treated blood-feeder (Table 2).

Table 1. Repeated G test goodness-of-fit test, percent response per trial and mean percent ± SE across trials of the response of 5- to 8-days-old *Ae. aegypti* ([1]$n = 50–55$) adult females attracted to blood-feeders located on opposite sites of the 82 (L) × 45 (W) × 52 (H) cm Plexiglas cage during 15-min experiments at 25°C and 80% RH and treated with 0.05 μg/1 ml 95% ethanol (low dose) or the control (1 ml 95% ethanol).

			Percent (total number) mosquito response per trial	
	G value	*p* value	Treatment	Control
Trial 1	2.942	0.086	22.2% (2)	77.8% (7)
Trial 2	1.409	0.235	40.0% (14)	60.0% (21)
Trial 3	1.132	0.287	59.4% (19)	40.6% (13)
Trial 4	3.221	0.073	54.1% (20)	45.9% (33)
Total G	8.704	**0.069**		
Pooled G	2.809	0.094		
Heterogeneity G	5.895	**0.117**		
Mean ± SE			43.9% ± 8.3%	56.1% ± 8.3%

[1]Number of mosquitoes used in a trial; [2]total number of mosquitoes to respond.

Table 2. Repeated G test goodness-of-fit test, percent response per trial and mean percent ± SE across trials of the response of 5- to 8-days-old *Ae. aegypti* ([1]$n = 50–55$) adult females attracted to blood-feeders located on opposite sites of the 82 (L) × 45 (W) × 52 (H) Plexiglas cage during 15-min experiments at 25°C and 80% RH and treated with 0.50 μg/1 ml 95% ethanol (intermediate dose) or the control (1 ml 95% ethanol).

			Percent (total number[2]) mosquito response per trial	
	G value	*p* value	Treatment	Control
Trial 1	20.266	**6.739E-06**	76.1% (54)	23.9% (17)
Trial 2	21.745	**3.114E-06**	75.3% (61)	24.7% (20)
Trial 3	10.124	**0.001**	12.5% (2)	87.5% (14)
Trial 4	1.236	0.266	42.3% (22)	57.7% (30)
Total G	53.371	**7.127E-11**		
Pooled G	15.473	8.369E-05		
Heterogeneity G	37.898	**2.971E-08**		
Mean ± SE			51.5% ± 15.2%	48.5% ± 15.2%

[1]Number of mosquitoes used in a trial; [2]total number of mosquitoes to respond.

Table 3. Repeated G test goodness-of-fit test, percent response per trial and mean percent ± SE across trials of the response of 5- to 8-days-old *Ae. aegypti* ([1]$n = 50–55$) adult females attracted to blood-feeders located on opposite sites of the 82 (L) × 45 (W) × 52 (H) Plexiglas cage during 15-min experiments at 25°C and 80% RH and treated with 1.00 μg ml^{-1} 95% ethanol (high dose) or the control (1 ml 95% ethanol).

			Percent (total number) mosquito response per trial	
	G value	*p* value	Treatment	Control
Trial 1	5.004	**0.025**	65.4% (34)	34.6% (18)
Trial 2	13.903	**<0.001**	71.1% (54)	28.9% (22)
Trial 3	0.491	0.483	45.1% (23)	54.9% (28)
Trial 4	0.167	0.683	54.2% (13)	45.8% (11)
Total G	19.565	**<0.001**		
Pooled G	10.059	0.002		
Heterogeneity G	9.506	**0.02**		
Mean ± SE			58.9% ± 5.8%	41.1% ± 5.8%

[1]Number of mosquitoes used in a trial; [2]total number of mosquitoes to respond.

However, a significant difference in mosquito attraction to the treatment or control was determined between trials ($G_H = 37.898$, $df = 3$, $p = 2.971E-08$) (Table 2). There was a significant attraction to the treatment blood-feeder (~ 75% to the treatment) in trials one and two; however, there was a significant attraction (87.5%) to the control blood-feeder in trial three.

A significant difference in mosquito attraction to the treatment or control was determined between trials ($G_H = 9.506$, $df = 3$, $p = 0.02$) for mosquitoes tested with the high dose

(1.0 μg ml^{-1}, Table 3). Mosquitoes during trials one and two exhibited a significant level of attraction (65% and 71%) to the treated, rather than the control, blood-feeder (Table 3). Responses in the third and fourth trials were relatively equal responses to treatment (45% and 55%, respectively) and control (54% and 45%, respectively).

Comparison of mosquito attraction across doses shows a dose-dependent response to mycolactone

The log odds ratios of choosing a particular dose were calculated and showed mosquitoes were more attracted to the blood-feeder treated with the highest dose (1.0 μg ml^{-1}) of mycolactone rather than the low dose (0.05 μg ml^{-1}), indicating a dose response (Fig. 1). The odds of being attracted to the 0.05 μg ml^{-1} dose (0.832) were less than being attracted to the intermediate dose of 0.50 μg ml^{-1} (1.511). As with the G test of goodness-of-fit, replicate variance was significantly different from 0 and was included as a random effect in the final model. Dose ($p = 0.0289$) was a significant predictor of response. Odds of responding to the treatment were greatest (1.433) for 1.00 μg ml^{-1} dose, while the lowest odds (0.832) of response were for 0.05 μg ml^{-1} dose.

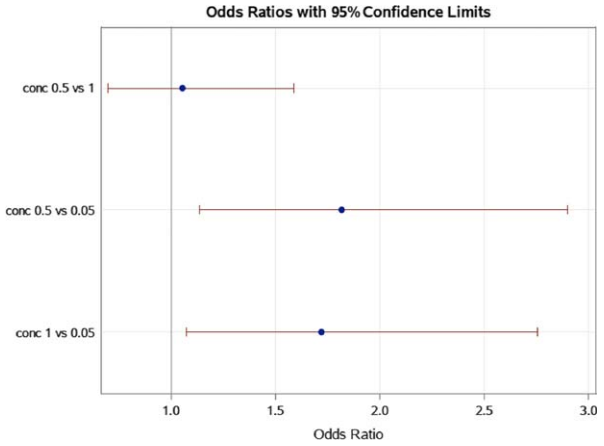

Fig. 1. Odds of choosing blood-feeder device by *Ae. aegypti* according to mycolactone dose.

Oviposition preference for sites treated with mycolactone

Percent attraction responses by *Ae. aegypti* adults to artificial oviposition sites treated with mycolactone at different concentrations are presented in Tables 4–6. At the low (0.05 μg ml^{-1}) and intermediate (0.50 μg ml^{-1}) doses, 41.6% and 38.3% were attracted to the treated oviposition sites respectively, indicating repellence by mycolactone. A significant difference in mosquito oviposition on treated or control sites was determined between trials for the low (0.05 μg ml^{-1}) ($G_H = 530.321$, $df = 3$, $p = 1.28E\text{-}114$) (Table 4) and intermediate (0.5 μg ml^{-1}) ($G_H = 11.5$, $df = 3$, $p = 0.009$) (Table 5) dose. For the intermediate dose (0.5 μg ml^{-1}), once again a significant attraction to the control oviposition site was found in all trials (~ 61.7%), indicating repellence. Overall mosquito response to the high dose (1.0 μg ml^{-1}) indicated marginal attraction (53.1% overall, 71.3% and 58.9% during trials three and four). A significant difference in mosquito oviposition on treated or control sites was determined between trials ($G_H = 247.316$, $df = 3$, $p = 2.491E\text{-}53$) (Table 6). At this dose, mosquito response in trials three and four indicate attraction (~ 64%) to the treated oviposition site. However, mosquito response during trial one indicated random (51.5%) response while repellence during trial two (69.3%).

Comparison of mosquito oviposition across doses shows a dose-dependent response to mycolactone

Mosquitoes were more attracted to the oviposition site treated with the highest dose (1.0 μg ml^{-1}) of mycolactone rather than the lower doses (0.05 and 0.50 μg ml^{-1}).

Table 4. Repeated G test goodness-of-fit test, percent response per trial and mean percent ± SE across trials of the response of 5- to 8-days-old adult *Ae. aegypti* ([1]$n = 55$) attracted to filter paper in oviposition sites located on opposite sites of the 61 (L) × 61 (W) × 61 (H) mesh cage during 24-hr experiments at 25°C and 80% RH and treated with 0.05 μg ml^{-1} 95% ethanol (low dose) or the control (1 ml 95% ethanol).

			Percent mosquito eggs deposited	
	G value	*p* value	Treatment	Control
Trial 1	267.462	**4.056E-60**	32.4% (683)	67.6% (1426)
Trial 2	257.228	**6.899E-58**	27.9% (353)	72.1% (914)
Trial 3	88.075	**6.302E-21**	58.3% (1854)	41.7% (1326)
Trial 4	3.520	**0.061**	47.9% (1008)	52.1% (1094)
Total G	616.285	**4.63E-132**		
Pooled G	85.964	1.832E-20		
Heterogeneity G	530.321	**1.28E-114**		
Mean ± SE			41.6% ± 7.0%	58.4% ± 7.0%

[1]Number of mosquitoes used in a trial.

Table 5. Repeated G test goodness-of-fit test, percent response per trial and mean percent ± SE across trials of the response of 5- to 8-days-old adult *Ae. aegypti* ([1]$n = 55$) attracted to filter paper in oviposition sites located on opposite sites of the 61 (L) × 61 (W) × 61 (H) mesh cage during 24-hr experiments at 25°C and 80% RH and treated with 0.50 μg ml^{-1} 95% ethanol (intermediate dose) or the control (1 ml 95% ethanol).

			Percent mosquito eggs deposited	
	G value	*p* value	Treatment	Control
Trial 1	125.207	**4.585E-29**	36.5% (616)	63.5% (1073)
Trial 2	93.253	**4.602E-22**	35.2% (1218)	64.8% (1742)
Trial 3	103.059	**3.253E-24**	40.9% (1255)	59.1% (1816)
Trial 4	16.646	**4.505E-05**	40.6% (190)	59.4% (278)
Total G	338.165	**6.296E-72**		
Pooled G	326.665	5.119E-73		
Heterogeneity G	11.5	**0.009**		
Mean ± SE			38.3% ± 1.4%	61.7% ± 1.4%

[1]Number of mosquitoes used in a trial.

Table 6. Repeated G test goodness-of-fit test, percent response per trial and mean percent ± SE across trials of the response of 5- to 8-days-old adult *Ae. aegypti* ([1]$n = 55$) attracted to filter paper in oviposition sites located on opposite sites of the 61 (L) × 61 (W) × 61 (H) mesh cage during 24-hr experiments at 25°C and 80% RH and treated with 1.00 μg ml^{-1} 95% ethanol (high dose) or the control (1 ml 95% ethanol).

			Percent mosquito eggs deposited	
	G value	*p* value	Treatment	Control
Trial 1	1.043	0.307	51.5% (571)	48.5% (537)
Trial 2	79.924	**3.891E-19**	30.7% (161)	69.3% (363)
Trial 3	182.404	**1.45E-41**	71.3% (695)	28.7% (280)
Trial 4	53.255	**2.929E-13**	58.9% (969)	41.0% (674)
Total G	316.626	**2.804E-17**		
Pooled G	69.310	8.414E-17		
Heterogeneity G	247.316	**2.491E-53**		
Mean ± SE			53.1% ± 8.5%	46.9% ± 8.5%

[1]Number of mosquitoes used in a trial.

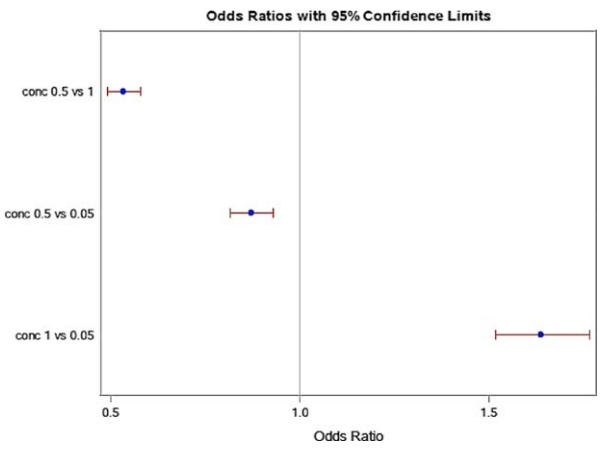

Fig. 2. Odds of choosing an oviposition site by *Ae. aegypti* based according to mycolactone dose.

The odds of being attracted to the 0.05 μg ml^{-1} (0.7598) dose were slightly higher than for being attracted to the 0.50 μg ml^{-1} (0.6617, Fig. 2) dose. Odds of responding to the treatment were greatest (1.2437) for the 1.00 μg ml^{-1} dose. Replicate was significantly different from 0 and was included as a random effect in the final model. Dose ($p < 0.0001$) was a significant predictor of response.

Discussion

This study is the first to demonstrate a potential biological role of mycolactone in aquatic environments. We demonstrated specifically that mycolactone could serve as a mechanism regulating attraction of *Ae. aegypti* adults to a potential blood-meal, and the response was dose dependent. This discovery, though novel in the MU-BU field, should not come as a surprise considering the number of arthropods that have been shown to use microbial products to communicate and locate resources (Davis *et al.*, 2013). Attractiveness of mosquitoes to a host is influenced by chemical cues, with CO_2 being among the most important (McMeniman *et al.*, 2014; Omondi *et al.*, 2015); however, more recent studies have shown that volatiles released by bacteria can cause a change in mosquito behaviour (Verhulst *et al.*, 2009). For example, volatiles released by *Staphylococcus epidermidis*, which resides on

human skin, can attract mosquitoes (Verhulst *et al*., 2009). *Anopheles gambiae*, Giles (Diptera: Culicidae), which transmits *Plasmodia* species responsible for malaria, display variable (differential) attractiveness to humans, depending on different compositions of skin microbiota (Verhulst *et al*., 2011). Chemical differences between volatiles emitted can have an altered effect on the level of attractiveness to mosquitoes (Logan *et al*., 2008). Bacteria associated with host skin have been shown to release volatiles that attract adult mosquitoes to blood-meal sources (Takken and Verhulst, 2013). In some cases, the response of mosquitoes to bacteria appears to be species specific where some bacterial species are less attractive than others (Verhulst *et al*., 2010b). However, the ecological relevance of such information is not yet clear (Verhulst et al., 2010a,b). With regards to MU, mycolactone has a very low vapour pressure and high boiling point, so degradation products may volatilize. Additionally, mycolactone is UV active, so attractiveness from the mosquito could instead be due to visual cues in conjunction with olfaction (Beehler *et al*., 1993; Fidanze *et al*., 2001).

In this study, mycolactone served as an attractant at the high dose (1.0 μg ml^{-1}), whereas it functioned as a repellent at low doses (0.05 and 0.50 μg ml^{-1}). Higher concentrations (1.0 μg ml^{-1}) were attractive while the low concentration (0.05 μg ml^{-1}) was repellent. The dose-dependent shift in attraction from the low dose (0.05 μg ml^{-1}) to the high dose suggests that the high dose could be the biologically relevant dose. Unfortunately, little is known about naturally occurring mycolactone concentrations. MU DNA has been quantified from water, soil, biofilm, invertebrates, macrophytes and other matrices by PCR (Kotlowski *et al*., 2004; Williamson *et al*., 2008; Vandelannoote *et al*., 2010; Williamson *et al*., 2012; McIntosh *et al*., 2014), providing a better understanding of MU presence and abundance in aquatic habitats. Furthermore, very little is known about the expression of mycolactone. Whether mycolactone is produced in aquatic environments remains to be seen and, if so, under what conditions and whether the level of production is similar to that found under laboratory conditions.

Bacteria are known to influence oviposition site selection by mosquitoes (Ponnusamy *et al*., 2008b). This relationship has been demonstrated specifically for *Ae. aegypti*, and is confluent with the findings of the study. Previous efforts have demonstrated that bacterial communities associated with different oviposition substrates' (bamboo vs. white-oak) infusions differed with many belonging to the Proteobacteria (Ponnusamy *et al*., 2008b) and the community makeup influenced level of mosquito oviposition (Ponnusamy *et al*., 2010). Additionally, the attraction of mosquitoes to these oviposition sites was governed by metabolic products produced by the Proteobacteria, specifically carboxylic acids (Ponnusamy *et al*., 2008b). Furthermore, while communities across larval mosquito habitats can vary greatly, their functional profiles were similar in terms of catabolic activity (Ponnusamy *et al*., 2008a), suggesting constraints with regards to cue diversification and mosquito attraction and oviposition.

Similar to those studies above whose results showed microbial metabolites enhance mosquito attraction and oviposition, we determined *Ae. aegypti* adults laid a significantly larger amount of eggs in sites treated with the highest concentration (1.00 μg ml^{-1}) of mycolactone (Table 6). However, as with the blood-feeding attraction assay, approximately 30% more eggs were deposited on the control than the treatment with the low (0.05 μg ml^{-1}) or intermediate (0.50 μg ml^{-1}) dose. These data indicate, as with attraction to a blood-meal, oviposition by *Ae. aegypti* could be repelled by the lower (0.05 and 0.5 μg ml^{-1}) mycolactone doses. Although no significant preference of oviposition to the sites treated with the low (0.05 μg ml^{-1}) or intermediate (0.50 μg ml^{-1}) doses was observed, an increase in oviposition preference to the site treated with the highest dose was observed. Thus, this compound could be an indicator to the mosquito that a given environment is an appropriate oviposition site. Studies focusing on gravid *Ae. aegypti* behaviour response in the presence of the two bacterial species *Acinetobacter calcoaceticus* and *Enterobacter cloacae* showed that twice as many females were attracted to sites in the presence of those tested bacteria (Benzon and Apperson, 1988). Furthermore, *Aedes* spp. lay their eggs singly on a moist substrate often adjacent to bodies of water, as well as areas known to contain floodwater (Clements, 1999). These environments may also be conducive to MU proliferation and mycolactone production, and previous studies have found MU DNA in similar environments, often where people spend a great deal of time washing, bathing or conducting agriculturally associated activities (Williamson *et al*., 2008; 2012). However, more research should be conducted to determine the implications these results have with respect to BU risk and incidence.

The overall shift in attraction from the control to the high dose mycolactone treatment for blood-feeding and oviposition behaviours suggests the possibility that mycolactone could be serving as a means of interkingdom 'communication'. Our research group has previously demonstrated that QS by bacteria regulates arthropod attraction and colonization of a resource. Initial research was conducted with a blow fly (Diptera: Calliphoridae) model. We were able to demonstrate that disruption of swarming (a QS response) by *Proteus mirabilis* (Ma *et al*., 2012) resulted in a 50% reduction in fly attraction and oviposition on agar inoculated with the bacteria. We have since demonstrated that inhibiting QS (i.e., quorum-quenching) of the commensal skin bacterium, *S. epidermidis*, resulted in an

approximate 30% reduction in mosquito attraction to a blood-meal (Zhang *et al*., 2015).

A better understanding of the functional roles of virulence determinants outside of host cells and also how these organisms regulate their virulence repertoire while occupying natural habitats outside of mammalian hosts is becoming increasingly important for human health and food safety. The fact that MU is an environmental pathogen and does not depend on the human host for survival suggests that mycolactone has an important role in the population biology and community ecology of the species (Marsollier *et al*., 2007). As previously stated, the macrolide structure of mycolactone suggests the possibility that the molecule may be an antagonist to bacteria with QS machinery or may serve as a regulator of secondary metabolism (Hashimoto *et al*., 2011; Romero *et al*., 2011), and work is currently underway to understand QS related, mycolactone mechanisms. Taken together, our data suggest that mycolactone may increase MU fitness under conditions where mycolactone functions as a mosquito attractant. If so, this could be a powerful strategy for dispersal of these bacteria to a more nutrient rich environment, or to eliminate microbial competition by alerting mosquitoes to feed on hosts or other environments with desirable bacteria (Wallace *et al*., 2010). Clearly, further experimentation is necessary to test these hypotheses in order to decipher the ecological underpinnings allowing MU persistence within its natural habitat. A better understanding of this process could also lead to the development of novel methods for prevention of pathogen transmission.

Finally, demonstrating that mycolactone is a cue used by mosquitoes to locate hosts could have large implications with regards to refining the vector competency model. Vector competence of mosquitoes has been shown to be influenced by both intrinsic and extrinsic factors (Hardy *et al*., 1983). The extrinsic factors take into consideration if a mosquito will come in contact with a host that is suitable for the pathogen or virus being transmitted, whereas the intrinsic factors play a role in host mosquito attraction and the ability of the mosquito to be infected with the pathogen itself (Hardy *et al*., 1983). The influence microbes have on the ability of mosquitoes to transmit a pathogen provide potential to reduce the capacity of the vector, or enhance the ability to transmit the pathogen in both laboratory and wild-caught populations (Weiss and Aksoy, 2011). Commensal microbes have host–symbiotic relationships that, in most cases, enhance vector-competence originating in nature, often picked up in the environment (Weiss and Aksoy, 2011). We have data detecting MU DNA on human skin in MU/BU endemic areas in West Africa (data not shown). Therefore, significant attraction to mycolactone in host-seeking behaviour could indicate that MU contamination from the environment on human skin could enhance mosquito attraction to a host, thus potentially increasing the capacity of the mosquito as a vector of the pathogens responsible for yellow fever, dengue fever, Zika or Chikungunya.

The findings presented indicate that there is some attraction to mycolactone in both of the tested scenarios; however, this attraction cannot be used to infer that *Ae. aegypti* are serving as the mode of MU transmission. Further studies are necessary to determine whether mosquitoes that come into contact with mycolactone and MU are able to transmit the pathogen to another host. Additionally, studies determining the level of attractiveness from the mosquito to mycolactone or viable MU found on skin are worthwhile. The more we know of the strategies used by one environmental pathogen, such as MU, the better we may understand whether similar strategies might exist and be used by other pathogens, the better we may understand the emergence and evolution of virulence determinants, and the more we may be able to control human infection rates and predict outbreaks of disease.

Materials and methods

Mosquito colony maintenance

Aedes aegypti aegypti colony was maintained in an incubator at 25°C, 80% RH and 12:12 L:D. Mosquito eggs (Liverpool strain) on a filter paper were placed in a container with 1 L of deionized water and allowed to hatch. Two-day-old larvae were separated into containers at a density of approximately 100–200 larvae/L in order to provide proper conditions for larval growth (Paskewitz, 1995). Larvae were fed a diet of fish food (TetraMin diet by Tetra Blacksburg, VA) that was sprinkled on the surface of the water. Food was provided every other day to prevent bacterial contamination of the water. Pupae were separated into 60 ml containers containing deionized water at an approximate density of 50 females/cup. Plastic cups containing approximately 50 pupae were then placed inside a 4.9 L grease-resistant paper bucket (Solo Cup Operating Corporation, Lake Forest, IL) with mesh coverage and allowed to emerge and mate. Newly emerged adults were provided a 5% sucrose solution via a damp cotton ball every other day until 24 hours prior to the blood-feeding experiment.
Mating

Mosquitoes were blood-fed approximately 5–8 days after emergence. In order to feed them, a blood-feeder was created using a 4.5 $\times$ 3 $\times$ 9 cm, 45 ml cell culture flask (Corning Incorporated, NY). In order to create a space gap to pipette blood used in this experiment, parafilm covered the surface of the blood-feeder (Fig. 1). The blood-feeder was then connected to a water bath maintained at 37°C. Mosquitoes were allowed to feed for approximately 3–4 h. Approximately three days after blood-feeding, a 2 $\times$ 5 cm filter paper placed in a 50 ml cup containing 30 ml of water was placed in the cage. Females were allowed to deposit eggs for 3 days. The filter paper containing eggs was then placed on a shelf in the incubator room, allowed to air dry and then stored until use.

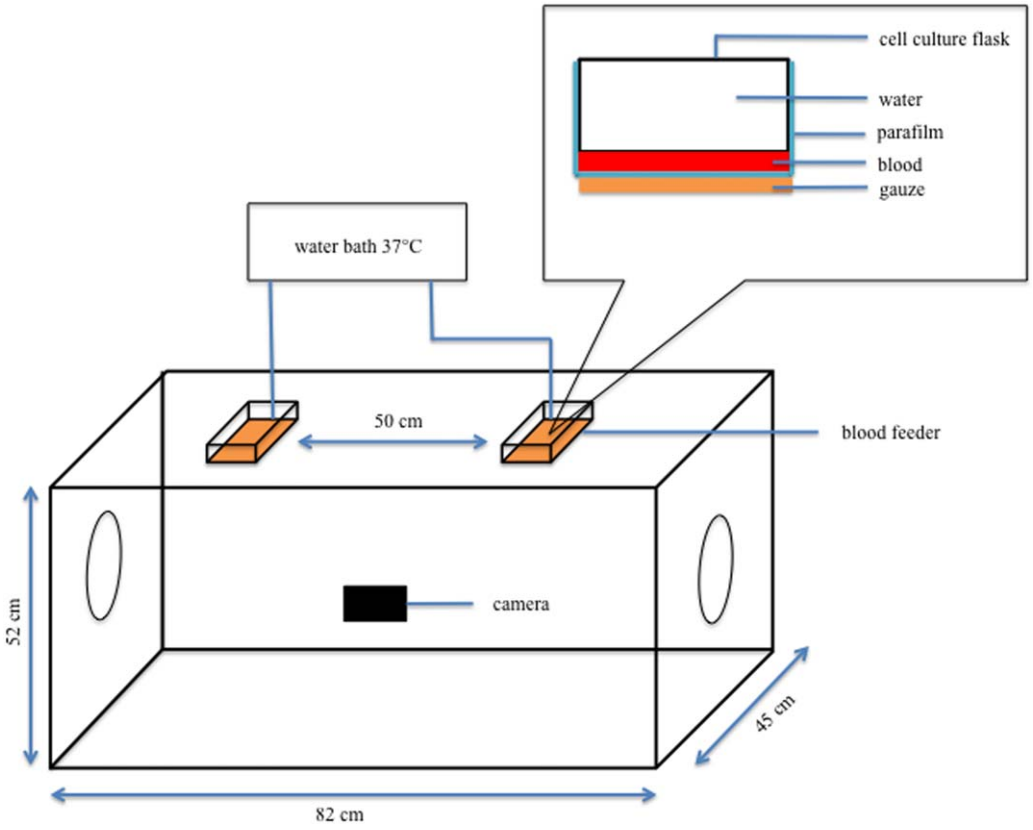

Fig. 3. The blood-feeders used to conduct the behavioural assays with 5- to 8-days-old *Ae. aegypti* in the device (adapted from Zhang *et al.*, 2015).

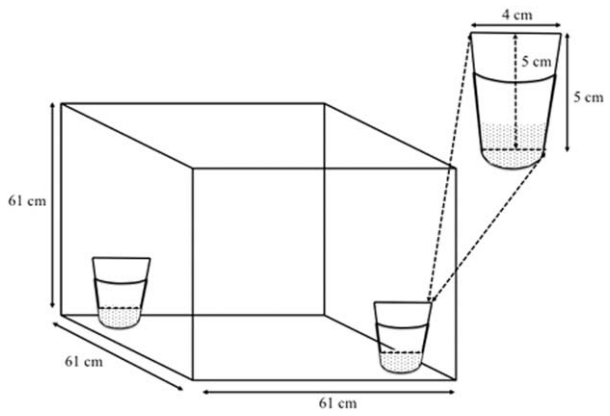

Fig. 4. The device used to conduct the oviposition assays with 5- to 8-days-old *Ae. aegypti*.

Mycolactone

Mycolactone A/B was isolated methods described by Mve-Obiang *et al.* (2003); however, the methods were altered where *M. ulcerans Agy*99 were grown on M7H10 plates + OADC instead of in broth, and bacteria were scraped from the plates and weighed. Methods for purifying mycolactone were as previously described (Mve-Obiang *et al.*, 2003), and a cytopathicity assay was conducted to confirm activity as previously described (George *et al.*, 2000). Mycolactone concentration was extrapolated from MU cell weight and corresponding colony count, where one cell was estimated to produce approximately 1 pg of mycolactone. Subsequent mycolactone doses used in this study were derived from using this information as well as MU qPCR values from environmental samples ranging from 1×10^3 to 1×10^6 GU ml^{-1} to obtain biologically relevant doses (Williamson *et al.*, 2008, 2012; McIntosh *et al.*, 2014). Mycolactone was desiccated after extraction and, at the time of use, 10 ml of 95% ethanol was added to each vial to solubilize samples used for the study to three concentrations: 1.00, 0.50 and 0.05 $\mu g\ ml^{-1}$. Ethanol (95%) was used as a control. Due to the negative effects of ultraviolet rays, mycolactone was stored in amber vials in a dark location to maintain sample stability.

Blood-feeding attraction experiment design

As these experiments rely on observing female behaviour, male and female pupae were sorted based on size and housed in separate containers previously described. The adult mosquitoes were sexed using physical characteristics, with the females antenna being bare compared with the plumose antenna characteristic of the males Gopfert *et al.* (1999). Resulting 5- to 8-days-old female mosquitoes were sedated with CO_2 (Scott *et al.*, 1993) and placed into a Plexiglas cage

(Fig. 1) held in the incubator room under previously described. The age group (5–8 days old) was chosen to allow mosquitoes the optimal age for conducting the behaviour experiments, as 3–4 days are needed to secure the mosquito numbers to conduct the experiments. Older age groups were excluded from the experiments, as they tend to be less selective. The mosquitoes were placed in the cage 30 minutes prior to the assay in order to allow them to acclimate. Between replications, the introduction of the mosquitoes consisted of alternating from the left to right side of the cage in order to prevent any bias. Attraction to light, air flow and CO_2 required acclimation to the environment 30 minutes prior to the blood-feeding to reduce the potential for positional bias (Turlings *et al*., 2004). In each replicate, two blood-feeders were used in order to provide a true choice between treatment and control.

Blood-feeders were placed parallel to one another on the opposite ends of the cage top, separated by approximately 50 cm. Both blood-feeders were connected by thin plastic tubing to a water bath set at 37°C to simulate the temperature of human blood as previously described (Pennes, 1948). Each blood-feeder was covered with 4-pleated gauze (Johnson & Johnson, New Brunswick, NJ) soaked with mycolactone or ethanol. An aliquot of 1 ml of rabbit blood (HemoStat Laboratories, Dixon, CA) was pipetted in the gap between the blood-feeder and the parafilm (Fig. 1). Preliminary experiments indicated blood-feeders treated with ethanol were not repellent to mosquitoes.

The numbers of mosquitoes feeding on either the treatment feeder or the control feeder were recorded using a high-definition camera (GoPro Inc., San Mateo, CA) that was mounted on the side of the Plexiglas cage according to established methods (Zhang *et al*., 2015). The videos were reviewed to count the sum of mosquitoes present on each individual blood-feeder every minute during a 15-minute assay period, with their presence on the blood-feeder being considered host-seeking activity. Each experiment was replicated four times, alternating the treatment blood-feeder between right and left ends of the cage top to rule out any positional effects.

Oviposition attraction experiment design

For these experiments, fifty-five 5- to 8-days-old blood-fed mosquitoes were placed into a 61 cm × 61 cm × 61 cm Plexiglas cage with a wire mesh top. Two small plastic cups approximately 5 cm in height were placed in the cage (Fig. 2), each containing a strip of 4 cm × 5 cm filter paper to be used as an oviposition substrate. In order for the substrate to be fit for oviposition, approximately 30 ml of deionized water was placed in the cup to reach the bottom of the filter paper. Prior to placing the filter paper in the cups, one strip was treated with 1 ml of 95% ethanol as a control and allowed to dry. The other strip of filter paper was treated with 1 ml of the desired mycolactone dose and allowed to dry. The two cups were placed in the 61 cm × 61 cm × 61 cm wire mesh cage and placed 50 cm apart at opposite corners of the cage (Fig. 2). For each replicate, the cups were rotated clockwise to prevent the position of the cup from creating a bias. The experiment consisted of four trails per dose of mycolactone tested. The conditions of the incubator were the same as for colony maintenance. The females were allowed 48 hours to oviposit and the numbers of eggs were counted from each piece of filter paper after the assay was completed. Preliminary experiments indicated oviposition sites treated with ethanol were not repellent to mosquitoes.

Statistical analysis

Each attraction assay was replicated four times and analysed using methods described by Tomberlin *et al*. (2012). A G test goodness-of-fit ($p \leq 0.05$) was used to determine the mosquito response between treatment and controls across treatments. This approach determined if the response was different from an expected ratio (50:50). Furthermore, unlike chi-square, this approach allowed us to determine if trial was a significant factor with regards to mosquito response (Zhang *et al*., 2015). A comparison of mosquito responses across doses was conducted with PROC GLIMMIX SAS 2011, a generalized linear mixed model (GLMM). The probability (p) of response (i.e., attraction and oviposition) by *Ae. aegypti* to the different mycolactone treatments was examined for significant difference ($p < 0.05$) across dose. Replicate was included in the model as a random factor (Figs. 3 and 4).

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