國立臺灣大學生物資源暨農學院昆蟲學系

碩士論文

Department of Entomology

College of Bioresources and Agriculture

National Taiwan University

Master Thesis

環境微生物對埃及斑蚊（雙翅目：蚊科）幼蟲存活率與成蟲產卵行為之影響

Effects of Environmental Microbes on *Aedes aegypti* (Diptera: Culicidae) Larval Survival and Oviposition

賴學濂

Hsueh-Lien, Lai

指導教授：薛馬坦 博士

Advisor: Matan Shelomi, Ph.D.

doi:10.6342/NTU202001573

Acknowledgements

首先要感謝我的上帝，謝謝祂帶領我走這條路，並給予我許多恩典得以順利走到最後；感謝指導教授 Matan Shelomi 在這三年間的許多教導、協助與關心；感謝實驗室舒慶渝同學、江旻柔學妹、黃詠恩學弟各種方面的協助；感謝我的家人們三年間持續的鼓勵與幫助；感謝翁淩維學姊與溫松翰學長在我碩一時的養蚊教學；感謝口試委員郭奇芊副教授、莊定武副教授、黃旌集博士對於我論文的指教；更感謝我的女友邱雅欣給我不間斷的支持與陪伴；最後將一切榮耀與成果獻給神。

doi:10.6342/NTU202001573

中文摘要

埃及斑蚊(*Aedes aegypti* (L.))等蚊類是茲卡、登革熱等蚊媒傳染病的病媒。在蚊子所產卵的水中有些微生物，這些微生物可以影響蚊子，例如影響蚊子產卵選擇或是可殺幼蟲，但大多數的環境微生物都還沒被測試過。本研究主要針對本實驗室在 2017 年於南台灣幾處可能適合蚊子產卵的水域採集的微生物，並以埃及斑蚊三齡幼蟲為實驗對象。以那些沒有蚊子存在的採集處之微生物，將高濃度的微生物放進幼蟲生長的水中並以蘇力菌作為正向控制，於 24 小時後觀察幼蟲的存活率。另外，將高濃度的微生物放進成蟲產卵容器中，觀察微生物是否影響成蟲的產卵行為。結果顯示，本研究測試的微生物皆無法造成幼蟲死亡或是影響成蟲產卵。這些微生物被採集時沒有幼蟲出現，可能是微生物與成蟲仍有未知的關係。原因可能在無法培養的菌或是與微生物無關，未來仍需探討此可能性。

關鍵詞：埃及斑蚊、殺幼蟲劑、產卵行為、細菌、微生物生態學。

doi:10.6342/NTU202001573

Abstract

Mosquitoes such as *Aedes aegypti* (L.) are vectors of lots of severe diseases, like Zika and dengue fever. Microbes in the water where mosquitoes lay eggs can affect mosquitoes, such as by affecting adult oviposition choice or working as a larvicide. The effects of most environmental microbes on mosquitoes have not been tested. My research focuses on microbes collected from potential mosquito oviposition sites in Southern Taiwan in 2017. I used late third instar *Ae. aegypti* larvae as model insects. For every microbe, focusing on those cultured from mosquito-free containers, I put a high concentration into containers of water with larvae, then checked what happened after 24 hours, using *Bacillus thuringiensis* as a positive control. I also put the same microbes into containers of water to see if the microbes would affect mosquito oviposition behavior. The microbes I tested could not kill the larvae or attract/repel mosquito from laying eggs. The lack of mosquitoes from the containers where these microbes were collected might be due to some unknown relationship between microbes and adult mosquitoes, such as nonculturable microbes, or due to non-microbial aspects of the containers.

Key words: *Aedes aegypti*, larvicide, oviposition, bacteria, microbial ecology.

doi:10.6342/NTU202001573

Table of Contents

doi:10.6342/NTU202001573

List of Figures

doi:10.6342/NTU202001573

List of Tables

1

doi:10.6342/NTU202001573

Introduction

I. Vector control

Aedes aegypti is one of the most important vectors of mosquito-borne diseases (Navarro *et al.*, 2003), such as Zika, dengue, and yellow fever. Since we have no cure or effective vaccine for dengue (Hairi *et al.*, 2003), vector control is the only way for us to fight the disease. For this reason and because mosquitoes are very bothersome, scientists are trying to figure out how to reduce their populations.

Using insecticides to kill mosquitoes is a method in vector control. In malaria control, insecticide-treated bed nets can successfully reduce populations of *Anopheles* mosquitoes by killing adults that come to feed on humans (Raghavendra *et al.*, 2011). For *Aedes* mosquitoes, pirimiphos-methyl is used as a chemical adulticide (Chung *et al.*, 2001), and malathion as a chemical larvicide (Li *et al.*, 2018). However, chemical insecticides could cause some problems, like killing non-target creatures and polluting the environment (Lacey and Lacey, 1990). For environmental reasons, a growing movement exists to move towards biological control of mosquitoes.

Microbial insecticides are good alternatives to chemical insecticides because they can be highly selective for mosquitoes (Floore, 2006), without affecting other insects or vertebrates. Microbial insecticides are used to handle mosquito-related problems in many countries, such as malaria in Gambia (Majambere *et al.*, 2007), Ethiopia

doi:10.6342/NTU202001573

(Seyoume and Abate, 1997), and Kenya (Fillinger and Lindsay, 2006). The most common microbial insecticides are *Bacillus thuringiensis* (Goldman *et al*., 1986; Seyoum and Abate, 1997) and *Metarhizium anisopliae* (Paula *et al*., 2011). *Bacillus thuringiensis* was first described and isolated by Ishiwata and named by Berliner (Beegle and Yamamoto, 1992). Different strains of *Bacillus thuringiensis* are used as insecticides against different insects. For example, *Bacillus thuringiensis kurstaki* is used as a Lepidoptera insecticide and *Bacillus thuringiensis israelensis* is used as a Diptera insecticide (Thorne *et al.*, 1986).

Although microbial insecticides are effective, some scientists report that some mosquitoes have evolved resistance to them (Mulla *et al.*, 2003). Zahiri *et al.* (2002) proved that resistance to *Bacillus sphaericus* has appeared in *Culex pipiens*, where the LC_{50} of *Bacillus sphaericus* has increased in every generation. Yuan *et al.* (2000) found that *Culex quinquefasciatus* in Dongguan, China, developed resistance to *Bacillus sphaericus* strain C3-41 after it had been used to control the mosquitoes for over six years. If the present microbial insecticides are losing their effectiveness, then finding other effective microbial insecticides is necessary.

Another way to control mosquitoes other than killing the larvae is to prevent the oviposition of eggs. Mosquitoes typically lay their eggs in or near standing water (Sota *et al.*, 1994). Different species of mosquitoes prefer to lay their eggs in different types

doi:10.6342/NTU202001573

of standing water: *Culex* typically prefer polluted water (Omolade and Adetutu, 2018), *Anopheles* typically prefer sunlit and fresh pools (Sumba *et al.*, 2004), and *Aedes* typically prefer smaller containers of clean water (Dom and Ahmad and Ismail, 2013). Gravid *Ae. aegypti* prefer to lay eggs in artificial water containers near people, such as vases, flower plots, or waste tires (Wong *et al.*, 2011).

The distribution of *Ae. aegypti* can be influenced by the availability of suitable oviposition sites (Edman *et al.*, 1998). Many abiotic factors of a container and its location affect female oviposition choice, such as the material of the containers, temperature (Nguyen *et al.*, 2014), light (Haddow and Gillett, 1957), and humidity (Canyon *et al.*, 1999). Biotic factors such as the presence or absence of mosquitoes, natural predators, and food in the water itself also affect oviposition choice. Allan and Kline (1998) proved that *Ae. aegypti* are more likely to lay eggs in their larval rearing water and water with preexisting eggs. Blaustein and Kotler (1993) found that *Culiseta longiareolata* prefer oviposition sites with more food for the larva and shun sites containing predatory *Bufo viridis* tadpoles.

The microbial community of the water sources also plays a role in mosquito oviposition choice. The microbes in the water can interact with mosquito larvae in many different ways. Microbes can be pathogenic or insecticidal, as described above. Microbes can be food for the larvae (Merritt *et al.*, 1992). Microbes can stimulate eggs

doi:10.6342/NTU202001573

laid in the water to hatch (Ponnusamy *et al.*, 2011). Lastly, research has found that mosquito larvae cannot fully develop in completely microbe-free water: microbes from the environment colonize the larval digestive tract, and this is essential for their healthy development into adults (Coon *et al.*, 2014). Note that mosquitoes do not have obligate microbial symbionts, meaning many different species of bacteria are equally effective as gut symbionts.

Since microbes can influence larval survival and development, one expects female mosquitoes could be able to sense the presence of microbes in the water in one way or another, and make oviposition choices accordingly. If two containers are otherwise identical, mosquitoes may prefer to lay eggs in waters with a more favorable microbiome. This hypothesis has been tested by several researchers. Sumba *et al.* (2004) discovered that *Anopheles gambiae* prefer to lay eggs in oviposition sites with living microorganism from natural larval habitats than in clear water.

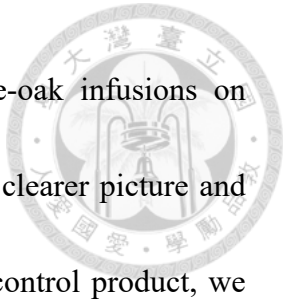
Based on these results, it may be possible to control the population of mosquitoes by taking advantage of microbial effects on their oviposition behavior. For example, one can make ovitraps that contain a microbe or microbial extract that attracts the mosquitoes to lay eggs and an insecticide to kill the larvae (Perich *et al.*, 2003). One can alternatively apply a repellent microbe to certain containers to keep mosquitoes from ovipositing. An attractant microbe could be genetically engineered to be larvicidal, or a

doi:10.6342/NTU202001573

larvicidal microbe can be engineered to be attractive, increasing its effectiveness in attract-and-kill ovitraps. Microbe-derived attractants may be used to make artificial lures, while microbe-derived repellents can be used as topical or spatial repellents for human use. One can even indirectly affect mosquitoes by killing essential microbes in the waters, either via antibiotics or via microbial antagonism using another microbe species as biocontrol.

A major obstacle to developing usable products or methods from microbes is that previous research did not typically match the effects of a microbiome with specific microbe species. For example, Sumba *et al.* (2004) found that *Anopheles gambiae* are attracted to the living microbiome from natural larval habitats and not sterilized microbes, but never tried to identify which microbe species were part of the microbiome. Yee *et al.* (2010) studied discarded tires in Illinois to find the relationship between mosquitoes and environmental factors, including bacterial biomass and protozoan abundance, but never did any microbe identification work. Yee *et al.* (2012) also examined the effects on mosquitoes of biomass and productivity of bacteria and fungi in tree holes and discarded tires in south Mississippi, but didn't identify the microbes. Kim *et al.* (2015) compared the bacterial composition and abundance from collected water samples and the gut microbiota of *Ae. japonicus*, *Ae. triseriatus* and *Cx. restuans*, but they only identified microbes to the phylum level, as did Ponnusamy *et al.* (2008) when

doi:10.6342/NTU202001573



they tested the effect of bacteria isolated from bamboo or white-oak infusions on mosquito oviposition. These results are starting points, but to get a clearer picture and certainly if we want to translate the results into a physical, vector-control product, we should try to identify microbes to species, if possible.

The goal of our laboratory project was to identify the microbiota of containers in the field with and without mosquito larvae, identify microbe species or clades that had a specific effect on mosquitoes, and confirm the existence of these effects in laboratory experiments. Our lab cultured microbes from potential *Aedes aegypti* breeding containers in south Taiwan in 2017 (Shelomi, 2019), and also used molecular profiling to identify microbes that could not be cultured. Our next objective was to test the cultured microbes for use in vector control.

Since vector control is presently the only way to deal with dengue, if the microbes I tested are larvicidal or can affect adult oviposition, then these microbes can potentially be used as new methods for vector control. I hoped to discover microbes with practical applications in vector management and hopefully lower the infection rate of dengue using these microbes. In the process, we could also learn more about the microbial ecology of bacteria in stagnant waters and how they interact with mosquitoes, and produce data that could be useful in future investigations, both basic and applied.

doi:10.6342/NTU202001573

II. Microbial Larvicide

Microbial larvicides are microbes that kill immature insects. Bacteria (Goldman *et al*., 1986), viruses (Federici, 1995), fungi (Paula *et al*., 2011), protozoa (Bell and McLaughlin, 1970), and nematodes (Nishimatsu and Jackson, 1998) can be used as microbial larvicides. These microbes typically have high specificity on target creatures and cannot hurt the human body, and some are even specific or at least preferentially pathogenic to aquatic Diptera or even to Culicidae.

1. Bacteria

Bacteria are commonly used as insecticides. The most widely used is *Bacillus thuringiensis* (Bt). After the larvae eat the bacteria, the bacteria are digested in the midgut and release crystals formed by these bacteria. The crystals are broken and release insecticidal proteins due to the alkaline environment of the midgut (Federici, 1995). These proteins can easily destroy an insect's midgut epithelium, then cause their death.

The different subspecies of Bt are well known larvicides for Lepidoptera (*Bacillus thuringriensis kurstaki*) and Diptera (*Bacillus thuringiensis israelensis*) insects. Cabrera *et al.* (2010) used Bt to control the tomato borer, *Tuta absoluta*. Kovendan *et al.* (2011) explored the possibility of using Bt to control the lymphatic filarial vector, *Cx. quinquefasciatus*. *Bacillus sphaericus* is another useful insect control agent.

doi:10.6342/NTU202001573

Kovendan *et al.* (2012) proved that *Bacillus sphaericus* has larvicidal and pupicidal activity when facing the malaria vector, *An. stephensi*. Davidson *et al.* (1984) found that *Bacillus sphaericus* strains 1593 and 2362 can control populations of *Cx. tarsalis*.

Though these bacteria are very effective at controlling insects, the existence of resistance is still a problem. McGaughey (1985) discovered *Plodia interpunctella* could develop resistance to Bt. Tabashnik *et al.* (1990) reported resistance in a field population of *Plutella xylostella* to Bt. Mosquitoes are also capable of developing resistance. Leroux *et al.* (1997) did laboratory selection experiments and found that *Culex pipiens* developed resistance to the *Bacillus sphaericus*. Rao *et al.* (1995) reported resistance in *Culex quinquefasciatus* to *Bacillus sphaericus* in the field in India.

2. Fungi

Fungi are another choice for microbial insecticides. Unlike other entomopathogens, fungi can infect and kill insects without being ingested. When the host contacts the spores, they will adhere to the cuticle and wait for a suitable environment to germinate. Then the cuticle will be destroyed by mechanical pressure and degrading enzymes (Thomas and Read, 2007). Some of the fungi kill the host by absorbing their nutrients, such as *Ophiocordyceps sinensis*, a valuable fungus in China (Wang and Yao, 2011). Others secrete toxic chemicals to kill the host. Fungi like

doi:10.6342/NTU202001573

Metarhizium anisopliae (Zimmermann, 1993) and *Beauveria bassiana* (Xu *et al.*, 2009) belong to this group. Fungi can be combined with chemical insecticides to be more effective. Paula *et al*. (2011) made the combination of *Metarhizium anisopliae* and Imidacloprid to kill adult *Ae. aegypti*, and the combination worked better than Imidacloprid only. Fungi can control malaria effectively. Blanford *et al*. (2005) used *Beauveria bassiana* and found that the fungus could not only kill the malaria vector *An. stephensi*, but also stop the malaria parasite, *Plasmodium chabaudi*, from developing into sporozoites, reducing the risk that the mosquito will transmit malaria before it dies.

Since pests are likely to develop resistance to microbial insecticides over time, in order to keep using microbes to deal with mosquitoes, it is necessary to discover new microbial insecticides that have never been used before. Yuan *et al.* (2000) found that resistance to *B. sphaericus* in *Cx. quinquefasciatus* could be eliminated after six months treatment with an alternative microbial insecticide. New microbial insecticides are needed to replace those lost to resistance, and to add to our current set of control methods. The possibility also exists that a novel microbial insecticide exists with superior qualities to those on the market today, such as better host specificity, lower LC_{50}, and/or faster mortality.

The microbes our lab collected from southern Taiwan included bacteria and fungi that can be cultured and maintained. Since some of them were only found in containers

doi:10.6342/NTU202001573

without mosquito larvae, the possibility exists that these microbes are larvicidal, killing larvae or eggs, as other authors have hypothesized when they found microbes limited to larva-free containers (Nilsson *et al*., 2018). I decided to test this hypothesis with experiment.

III. Adult Oviposition

The oviposition behavior of mosquitoes is another target of study in order to control mosquitoes. Mosquitoes' oviposition behavior can be affected by many abiotic factors of the container itself, such as its volume or the size of its opening (Harrington *et al.*, 2008), and whether it is in the shade or the light (Wong *et al.*, 2011). They are also affected by factors such as the amount of food for larvae in the selected site (Blaustein and Kotler, 1993), proximity to certain plants or to human habitation, or the existence of microbes in the water (Ponnusamy *et al.*, 2008).

Microbes play very important roles in mosquito oviposition behavior. Many scientists had reported that mosquitoes prefer to lay eggs in containers with microbes relative to microbe-free waters (Benzon and Apperson, 1988). Organic infusions like plant infusions, which are rich in microbes, can strongly induce mosquitoes to lay eggs (Ponnusamy *et al.*, 2010). In a laboratory experiment, *Ae. albopictus* and *Ae. aegypti* were more attracted to an infusion made with non-sterile leaves than that made with

doi:10.6342/NTU202001573

sterilized leaves, meaning the microbes on the leaves were responsible for the attractive effect rather than a chemical in the leaves themselves (Ponnusamy *et al.*, 2010). These results suggest that microbes strongly affect mosquitoes' oviposition behavior, however the actual microbes responsible for these effects were never identified in that study.

Not only complex microbial communities have the ability to affect mosquitoes, but also single microbes can have an effect. The existence of microbial insecticide Bti in a container affects the shape and the amount of eggs in *Cx. quinquefasciatus*' egg raft (Zahiri and Mulla, 2006). Effects on oviposition are not limited to microbial pathogens. *Ae. aegypti* prefers to lay eggs in a pure suspension of *Acinitobacter calcoaceticus* than a pure suspension of *Enterobacter cloacae* (Benzon and Apperson, 1988), neither of which is pathogenic. *Trichoderma viride*'s secondary metabolites can attract gravid *Cx. quinquefasciatus* (Geetha *et al.*, 2003).

The idea of controlling mosquito populations by their oviposition behavior is mainly through using the attract-and-kill method, by using an ovitrap loaded with baits and insecticides. The baits attract mosquitoes to lay eggs in the trap and the insecticides kill the larvae. The most commonly used insecticide in such traps is Bt. Carrieri *et al.* (2009) tested the effect of ovitraps with Bt on *Ae. albopictus'* oviposition. The result showed that after 14 days, the residual Bt could still kill 100% of larvae. Not many people have looked at microbial baits for ovitraps, however, and nearly all such research

doi:10.6342/NTU202001573

looked at microbe-rich plant infusions rather than single microbes. For example, Barbosa *et al.* (2010) used skatole and *Eleusine indica* (goosegrass) infusion to attract *Cx. quinquefasciatus* to lay eggs in ovitraps with Bti. Santos *et al.* (2003) used *Eleusine indica* infusion to attract gravid *Ae. aegypti* to lay eggs in ovitraps with Bti. No research on single-microbe attractants exist, but making ovitraps with such a microbe is much easier and more reliable than using plant infusions whose microbiota may be highly variable.

Alternatively, if a repellent microbe is discovered, it could be used as a non-lethal control to keep mosquitoes from ovipositing in certain containers. For example, adding the microbes to a permanent or semi-permanent container such as a cemetery flowerpot or a rain barrel could keep the container from becoming infested without the use of potentially harmful chemicals. Being nonlethal, this method would have few to no non-target effects on other organisms. The volatiles responsible for these repellents might even be extractable and used to make other repellent products such as personal, topical repellents or spatial repellents.

From among the microbes our lab collected, those collected from containers without larvae might have the ability to repel mosquitoes and/or discourage oviposition. I focused on these for this study. Should they have repellent or anti-oviposition

doi:10.6342/NTU202001573

activities, that would explain our observation of no larvae in these containers. Ideally, I could even find a microbe with potential use in vector management.

Materials and Methods

I. Mosquito Rearing.

The test insect used in this experiment is *Aedes aegypti*, provided by Professor Huang Rong-Nan from the Department of Entomology, National Taiwan University. The eggs are stored on paper towels in a sealed plastic box to keep them dry. To hatch the eggs, they are put into a glass bowl with dechlorinated reverse osmosis water (RO water). The bowl is then put into a vacuum environment made by a vacuum pump for one day. The larvae hatched from the eggs are housed in plastic pots (36.5 x 28.5 x 8.2 cm^3) containing two liters of RO water and feed with one spoon of commercial Flowerhorn fish feed pellets every day. After the larvae become pupae, the pupae are picked into a plastic cup (140 ml) containing 100 ml of RO water and placed in a BugDorm™ (MegaView Science Co., Ltd., Taiwan) plastic insect rearing cage (30.0x30.0x30.0 cm^3). The adults are fed with 10% sucrose solution. Both larvae and adults are housed in a growth chamber with an ambient temperature from 26.5°C to 27.5°C, a photoperiod of 12 L: 12 D, and a relative humidity of 70%.

doi:10.6342/NTU202001573

II. Microbe Larvicidal Test

The strains used in the experiments were all collected from different water containers in Tainan city, Kaohsiung City, or Pingtung City, Taiwan (Table 3.1). They were isolated on nutrient agar (HiMedia Laboratories, Mumbai, India) and were identified to species by 16S rRNA and ITS rRNA region sequencing (Shelomi, 2019). All the microbes were stored as glycerol stocks in a -80° C freezer, however only some survived this process. From those, the microbes chosen for larvicidal testing were those only found in containers with no *Aedes aegypti* larvae (Shelomi, 2019). *Bacillus thuringiensis* AM65-52 (VectoBac WDG, Abbott Laboratories, North Chicago, U.S.A) was tested as a positive control.

Microbes were plated onto nutrient agar one day before each test. For each microbe, a solution in RO water of $2.25*10^{10}$ colony forming units (CFU) per ml was made using fresh colonies, with microbe density estimated by matching the turbidity to that of a 0.5 McFarland Turbidity Standard. For the test, 25 late-third instar larvae were picked into a plastic cup (140 ml) containing 85 ml of RO water and 15 ml of the microbe solution, for a final microbial density of $2.25*10^{8}$ CFU/ml. This is a sufficient concentration to test the effects of a microbe on larval survival with little chance of a false negative (Elçin, 1995). After 24 hours, the number of surviving larvae was counted. Then all the larvae were moved to the glass bowl for rearing. The number of

doi:10.6342/NTU202001573

pupae and adults that emerged was counted. Two to four replicates were done for each microbe species depending on the availability of sufficient larvae.

III. *Mosquito Oviposition Test*

For the oviposition tests, eighty gravid adults were moved from the rearing cages to eight experimental cages. Each cage had ten adults. Two 140 ml plastic cups were set at the diagonal corners of each cage. One had 100 ml of RO water and the other had 85 ml of RO water and 15 ml of the microbe solution as described previously, for a final microbial density of $2.25*10^8$ CFU/ml. Both cups had a paper towel on the sides on which adults could lay eggs. Twenty-four hours after adding the cups, the paper towels with the mosquito eggs were taken out. The number of eggs on the microbe paper towel and the control paper towel were counted and compared with each other. The number of eggs in microbe or control cups in each replicate bioassay cage were converted to the proportion of the total number of eggs laid in the cage, then arcsine square root transformed to stabilize the variance of the binomial distribution and achieve normality (Ponnusamy *et al.*, 2008). The transformed proportions of eggs in the microbe and control cups were compared with a two-tailed, paired t-test.

The unforeseeable loss of microbe cultures due to the catastrophic failure of the -80°C freezer meant I could only test a limited number of microbes. These four

doi:10.6342/NTU202001573

microbes were *Fusarium delphinoides* and *Purpureocillium lilacinum* for fungi, and *Gordonia neofelifacis* and *Massilia arvi* for bacteria. These two bacteria were the only two bacteria from the original targets that could still be cultured, none with any connection in the literature to mosquitos. *Fusarium delphinoides* is a plant pathogenic fungus that can produce indole-3-acetic acid for the growth of root and shoot in plants (Kulkarni *et al.*, 2013). *Purpureocillium lilacinum* is an entomopathogenic fungus against cotton pests (Lopez *et al.*, 2014; Lopez and Sword, 2015).

The possibility exists that adult mosquitoes carried microbes from the microbe cups to the control cups, which would prevent detection of any effects of the microbe. To check for this and ensure that microbes survived in each container for the duration of the experiment, aliquots of water from each container, control and microbial, were plated onto nutrient agar and potato dextrose agar petri dishes and incubated for at least two days, with the hopes that the microbe water sample would contain the active microbe. Fungal colonies were identified by colony morphology and microscopy. Bacterial colonies were and identified by PCR and sequencing of the 16S gene.

doi:10.6342/NTU202001573

Table 1 The chosen microbes for the test.

Species	Collected Container	Bacteria or Fungi
Enterobacter tabaci	artificial	bacteria
Flavobacterium fontis	artificial	bacteria
Fusarium delphinoides	artificial	fungi
Gordonia neofelifaecis	plant	bacteria
Herbaspirillum aquaticum	artificial	bacteria
Massilia arvi	artificial	bacteria
Massilia albidiflava	artificial	bacteria
Nubsella zeaxanthinifaciens	artificial	bacteria
Purpureocillium lilacinum	artificial	fungi
Pseudomonas taiwanensis	artificial	bacteria
Serratia marcescens	ground	bacteria
Streptomyces misionensis	artificial	bacteria

doi:10.6342/NTU202001573

Results

I. Microbe Larvicidal Test

Depending on larval availability, I did two replicates for each microbe at first. Unfortunately, due to the -80°C fridge breaking down unexpectedly in 2019, most of the microbes intended for this experiment died. Therefore, only four surviving microbes were tested for four replicates.

Every larva survived in all twelve microbes' tests (0% mortality). Only the *Bacillus thuringiensis* positive controls had no larva survive (100% mortality). Given the results, no further statistical analysis was needed. The strength of these results is such that the number of replicates for each species is still sufficient to achieve a desired power ≥0.8 (alpha = 0.05) (Rosner, 2011).

II. Mosquito Oviposition Test

For all microbes tested, no significant differences in the number of eggs laid in microbe or control cups could be detected (Table 3). The results of the petri dish incubations confirmed that the fungi (*Fusarium delphinoides* and *Purpureocillium lilacinum*) could still be isolated from the microbe cups after 24 hours, and were not present in the control waters (Figure 1). Multiple bacteria species were present in the containers that initially had RO water only or RO water and a single microbe.

doi:10.6342/NTU202001573

Sequencing of colonies on these plates did not reveal *Gordonia neofelifacis* or *Massilia arvi* in either the microbe or control waters, however PCR failed for some of the colonies and they could not be identified. The microbes that could be identified had 16S sequences homologous to *Acinetobacter soli* and to an unidentified species of *Telmatobacter*.

doi:10.6342/NTU202001573

Table 2 The egg amount of the oviposition tests.

Species	Replicates	Control	Microbe
Fusarium delphinoides	1	96	92
	2	69	250
	3	0	415
	4	185	118
Gordonia neofelifacis	1	150	25
	2	104	163
	3	368	247
	4	293	80
Massilia arvi	1	11	304
	2	181	51
	3	77	77
	4	172	89
Purpureocillium lilacinum	1	139	178
	2	193	173
	3	321	329
	4	210	351

doi:10.6342/NTU202001573

Table 3 The statistics results of the oviposition tests.

Microbe	T(df)	P
Fusarium delphinoides	t(3)=1.1967	p>0.1
Gordonia neofelifacis	t(3)=1.5210	p>0.1
Massilia arvi	t(3)=0.1752	p>0.1
Purpureocillium lilacinum	t(3)=1.2396	p>0.1

doi:10.6342/NTU202001573

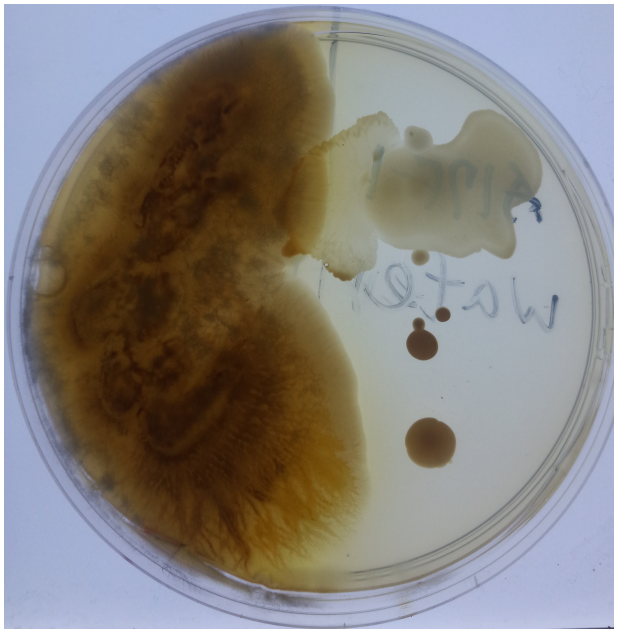


Fig. 1 A petri dish (potato dextrose agar) containing water from the microbe cup (left half) and RO water cup (right half). The microbe in this instance was *Fusarium delphinoides*, easily visible growing in the microbe half of the plate only. Unknown species of microbes are growing in the RO water section. They are likely environmental contaminants.

doi:10.6342/NTU202001573

Discussion

None of the tested microbes collected from the mosquito-free containers in southern Taiwan showed any larvicidal activity. The results are unlikely to be false negatives: My methods are based on the World Health Organization's gold-standard guidelines for testing microbial larvicides (WHO, 2005), and my positive control successfully killed off the larvae under the same conditions. My research used higher amounts of bacteria than most published studies (Ejiofor and Okafor, 1989; Davidson *et al.*, 1984), meaning my lack of observed larvicidal activity cannot be due to too low microbe concentrations. While the possibility exists that they are slow-acting pathogens and would have caused mortality if we waited longer, such a long wait is not within the accepted guidelines for finding larvicides (WHO, 2005), and such a slow-acting larvicide relative to Bt would have limited or no value in pest management.

From these twelve microbes, only *Serratia marcescens* had been previously tested for larvicidal ability, and was found to be larvicidal against mosquitoes (Patil *et al.*, 2011). However, my research used whole, living microbes to do the test, while Patil *et al.* isolated the pigment of *Serratia marcescens* and tested its larvicidal ability alone. Exposure to the isolated pigment might cause larval death, but not the living microbes.

Although none of the microbes can kill *Ae. aegypti* larvae, some of them still work as insecticides or have other effects. Pailan *et al.* (2015) found a degradation

doi:10.6342/NTU202001573

pathway of parathion, which is a widely used insecticide in India, in *Bacillus aryabhattai* strain SanPS1. *Purpureocillium lilacinum* was found to be an entomopathogenic fungus of cotton aphid, *Aphis gossipii* (Lopez *et al.*, 2014), and cotton bollworm, *Helicoverpa zea* (Lopez and Sword, 2015). Chen *et al.* (2014) reported that *Pseudomonas taiwanensis* was an entomopathogenic bacterium of some agricultural pests, like *Pletulla xylostella*.

For the oviposition test, none of the microbes tested significantly affected female oviposition positively or negatively. Same as in the larvicidal test, the amounts of microbes I used were higher than other microbial oviposition tests before (Ponnusamy *et al.*, 2010). Fungi were successfully re-cultured from the microbe water after the experiment concluded, and I found no evidence that they had been carried from the microbe water to the control water (Figure 1). I thus conclude that the results for the fungi are not false negatives, and that these two fungi are neither attractant nor repellent.

All the water samples including the controls contained several species of bacteria, which were not the two species added to the cups: *Acinetobacter soli* and an unidentified species of *Telmatobacter*. However, PCR amplification of the 16S gene failed for some of the extracted colonies, so the possibility exists that these were the original bacteria. Nonetheless, both the microbe and control waters had similar culturable microbes. The mosquitoes themselves may have brought bacteria to the cups,

doi:10.6342/NTU202001573

or bacteria from the air contaminated both cups equally. Because I cannot be sure if either *Gordonia neofelifacis* or *Massilia arvi* were present in and only in the microbe cups, I cannot be certain of their results and draw no conclusions over whether or not the affect adult mosquito oviposition behavior.

None of these four microbes were tested for their ovipositional activity before. Therefore, even though the null hypothesis was supported, the results are still useful for others to know that these fungi at least do not affect mosquito oviposition behavior, as this information can make further interpretation of microbial ecology work easier.

Conclusion

Our lab collected microbes from southern Taiwan. Some of them only appeared in containers without mosquito larvae. Those microbes were hypothesized to be larvicides or to have the ability to affect mosquito oviposition. Since the microbes were collected from dengue-endemic southern Taiwan, I chose the dengue vector *Aedes aegypti* as the model insect to do the tests. Despite the loss of many cultured microbes, I could still test 12 microbes for larvicidal ability and four microbes for oviposition effects. None of the microbes tested can kill the larvae or affect mosquito oviposition. Negative results are still results, and this experimental data helps us better understand how the microbial

doi:10.6342/NTU202001573

community of the containers can affect mosquito behavior and larval survival. At the very least, we can narrow down the likely microbes to a smaller list.

The microbiota of these containers differed greatly. Microbe communities in containers change over time, as others have noted (Ponnusamy *et al.*, 2008). The presence of larvae themselves can alter the microbiome (Yee *et al.*, 2007). For example, if an attractant microbe is also the larva's preferred food source, its population could have fallen to zero before we investigated. The possibility also exists that the microbes with the main effects on the mosquitoes are not culturable, and are among the OTUs identified in microbiome profiling but not available for testing.

There might still be some way that the collected microbes from southern Taiwan can affect mosquitoes. Some microbes were found only in containers with larvae or had been identified as indicators for them. These species might be attractants or oviposition stimulants and can be tested for these abilities in the future. In addition, these microbes might have other relationships with larvae, such as controlling their growth, serving as food, or changing containers; environments. While microbiome analysis can provide leads into such research, ultimately there is no substitute for culturing microbes and testing them directly, even if it is labor intensive, as that is the best way to be certain of a true effect.

doi:10.6342/NTU202001573

References

Allan SA, Kline DL. 1998. Larval rearing water and preexisting eggs influence oviposition by *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae). Journal of Medical Entomology 35(6): 943-947.

Barbosa RMR, Regis L, Vasconcelos R, Leal WS. 2010. *Culex* mosquitoes (Diptera: Culicidae) egg laying in traps loaded with Bacillus thuringiensis variety israelensis and baited with skatole. Journal of Medical Entomology 47(3): 345-348.

Beegle CC, Yamamoto T. 1992. Invitation paper (C.P. Alexander fund): history of *Bacillus thuringiensis* Berliner research and development. The Canadian Entomologist 124(4): 587-616.

Bell MR, McLaughlin RE. 1970. Influence of the protozoan *Mattesia grandis* McLaughlin on the toxicity to the boll weevil of four insecticides. Journal of Economic Entomology 63(1): 266-269.

Benzon GL, Apperson CS. 1988. Reexamination of chemically mediated oviposition behavior in *Aedes aegypti* (L.) (Diptera: Culicidae). Journal of Medical Entomology 25(3): 158-164.

doi:10.6342/NTU202001573

Blanford S. Chan BHK, Jenkins N, Sim D, Turner RJ, Read AF, Thomas MB. 2005. Fungal pathogen reduces potential for malaria transmission. Science 308(5728): 1638-1641.

Blaustein L, Kotler BP. 1993. Oviposition habitat selection by the mosquito, *Culiseta longiareolata*: effects of conspecifics, food and green toad tadpoles. Ecological Entomology 18(2): 104-108.

Cabrera JG, Molla O, Monton H, Urbaneja A. 2011. Efficacy of Bacillus thuringiensis (Berliner) in controling the tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). BioControl 56: 71-80.

Canyon DV, Hii JLK, Muller R. 1999. Adaptation of *Aedes aegypti* (Diptera: Culicidae) oviposition behavior in response of humidity and diet. Journal of Insect Physiology 45(10): 959-964.

Carrieri M, Masetti A, Albieri A, Maccaganai B, Bellini R. 2009. Larvicidal activity and influence of *Bacillus thuringiensis* var. *israelensis* on *Aedes albopictus* oviposition in ovitraps during a two-week check interval protocol. Journal of the American Mosquito Control Association 25(2): 149-155.

Chen WJ, Hsieh FC, Hsu FC, Tasy YF, Liu JR, Shih MC. 2014. Characterization of an insecticidal toxin and Pathogenicity of Pseudomonas taiwanensis against insects. PLoS Pathogens 10(8): e1004288.

doi:10.6342/NTU202001573

Chung YK, Phua SGL, Chua YT, Yatiman R. 2001. Evaluation of biological and chemical insecticides mixture against *Aedes aegypti* larvae and adults by thermal fogging in Singapore. Medical and Veterinary Entomology 15(3): 321-327.

Coon KL, Vogel KJ, Brown MR, Strand AR. 2014. Mosquitoes rely on their gut microbiota for development. Molecular Ecology. 23(11): 2727-2739.

Davidson EW, Urbina M, Payne J, Mulla MS, Darwazeh H, Dulmage HT, Correa JA. 1984. Fate of *Bacillus sphaericus* 1593 and 2362 spores used as larvicides in the aquatic environment. Applied and Environmental Microbiology 47: 125-129.

Dom NC, Ahmad AH, Ismail R. 2013. Habitat characterization of *Aedes* sp. breeding in urban hotspot area. Procedia-social and Behavioral Sciences 85: 100-109.

Edman JD, Scott TW, Costero A, Morrison AC, Harrington LC, Clark GG. 1998. Aedes aegypti (Diptera: Culicidae) movement influenced by availability of oviposition sites. Journal of Medical Entomology 35(4): 578-583.

Ejiofor AO, Okafor N. 1989. Production of mosquito larvicidal *Bacillus thuringiensis* serotype H-14 on raw material media from Nigeria. Journal of Applied Microbiology 67: 5-9.

Elçin YM. 1995. *Bacillus sphaericus* 2362-calcium alginate microcapsules for mosquito control. Enzyme and Microbial Technology 17: 587-591.

doi:10.6342/NTU202001573

Federici BA. 1995. The future of microbial insecticides as vector control agents. Journal of the American Mosquito Control Association 11(2): 260-268.

Fillinger U, Lindsay SW. 2006. Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicides in rural Kenya. Tropical Medicine & International Health 11: 1629-1642.

Floore TG. 2006. Mosquito larval control practices: past and present. Journal of the American Mosquito Control Association 22: 527-533.

Geetha I, Paily KP, Padmanaban V, Balaraman K. 2003. Oviposition response of the mosquito, *Culex quinquefasciatus* to the secondary metabolite(s) of the fungus, *Trichoderma viride*.

Goldberg LJ, Margalit J. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. Mosquito News 37(3): 355-358.

Goldman IF, Arnold J, Carlton BC. 1986. Selection of resistance to *Bacillus thuringiensis* subspecies *israelensis* in field and laboratory populations of the mosquito *Aedes aegypti*. Journal of Invertebrate Pathology 47, 317-324.

Haddow AJ, Gillett JD. 1957. Observations on the oviposition-cycle of *Aedes* (*Stegomyia*) *aegypti* (Linnaeus). Annals of Tropical Medicine & Parasitology 51(2): 159-169.

doi:10.6342/NTU202001573

Hairi F, Ong CHS, Suhaimi A, Tsung TS, Ahmad MABA, Sundaraj C, Soe MM. 2003. A knowledge, attitude and practices (KAP) study on dengue among selected rural communities in the Kuala Kangsar District. Asia Pacific Journal of Public Health 15(1): 37-43

Harrington LC, Ponlawat A, Edman JD, Scott TW, Veymeylen F. 2008. Influence of container size, location, and time of day on oviposition patterns of the dengue vector, *Aedes aegypti*, in Thailand. Vector-Borne and Zoonotic Diseases 8(3): 415-424.

Kim CH, Lampman RL, Muturi EJ. 2015. Bacterial Communities and Midgut Microbiota Associated with Mosquito Populations from Waste Tires in East-Central Illinois. Journal of Medical Entomology 52(1): 63-75.

Kovendan K, Murugan K, Vincent S, Kamalakannan S. 2011. Larvicidal efficacy of *Jatropha curcas* and bacterial insecticides, *Bacillus thuringiensis*, against lymphatic filarial vector, *Culex quinquefasciatus* Say. Parasitology Research 109: 1251-1257.

Kovendan K, Murugan K, Vincent S, Barnard DR. 2012. Studies on larvicidal and pupicidal activity of *Leucas aspera* Willd. (Lamiaceae) and bacterial insecticide, *Bacillus sphaericus*, against malaria vector, *Anopheles stephensi* Liston. (Diptera: Culicidae). Parasitology Research 110: 195-203.

doi:10.6342/NTU202001573

Kulkarni GB, Sanjeevkumar S, Kirankumar B, Santoshkumar M, Karegoudar TB. 2013. Indole-3-acetic acid biosynthesis in Fusarium delphinoides strain GPK, a causal agent of wilt in chickpea. Applied Biochemistry and Biotechnology 169: 1292-1305.

Lacey LA, Lacey CM. 1990. The medical importance of riceland mosquitoes and their control using alternatives to chemical insecticides. Journal of American Mosquito Control Association 2: 1-93.

Leroux CN, Pasquier F, Charles JC, SinÈGre G, Gaven B, Pasteur N. 1997. Resistance to *Bacillus sphaericus* involves different mechanisms in *Culex pipiens* (Diptera: Culicidae) larvae. Journal of Medical Entomology 34(3): 321-327.

Li Y, Xu J, Zhong D, Zhang H, Yang W, Zhou G, Su X, Wu Y, Wu K, Cai S, Yan G, Chen XG. 2018. Evidence for multiple-insecticide resistance in urban *Aedes albopictus* populations in southern China. Parasites & Vectors 11: 4.

Lopez DC, Salzman KZ, Ramos MJE, Sword GA. 2014. The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) and *Beauveria bassiania* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLoS One 9(8): e103891.

doi:10.6342/NTU202001573

Lopez DC, Sword GA. 2015. The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (Gossypium hirsutum) and negatively affect survival of the cotton bollworm (Helicoverpa zea). Biological Control 89: 53-60.

Majambere S, Lindsay SW, Green C, Kandeh B, Fillinger U. 2007. Microbial larvicides for malaria control in The Gambia. Malaria Journal 6: 76.

McGaughey WH. 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis.* Science 229(4709): 193-195.

Merritt RW, Dadd RH, Walker ED. 1992. Feedind behavior, natural food, and nutritional relationships of larval mosquitoes. Annual Reviews of Entomology 37:349-376.

Mulla MS, Thavara U, Tawatsin A. 2003. Emergence of resistance and resistance management in field populations of tropical *Culex quinquefasciatus* to the microbial control agent *Bacillus sphaericus*. Journal of the American Mosquito Control Association 19: 39-46.

Navarro DMAF, Oliveira PESD, Potting RPJ, Fital SJF. 2003. The potential attractant or repellent effects of different water types on oviposition in *Aedes aegypti* L. (Dipt., Culicidae). Journal of Applied Entomology 127: 46-50.

doi:10.6342/NTU202001573

Nilsson LKJ, Sharma A, Bhatnagar RK, Bertillson S, Terenius O. 2018. Presence of Aedes and Anopheles mosquito larvae is correlated to bacteria found in domestic water-storage containers. FEMS Microbiology Ecology 94(6), fiy058.

Nishimatsu T, Jackson JJ. 1998. Interaction of insecticides, entomopathogenic nematodes, and larvae of the western corn rootworm (Coleoptera: Chrysomelidae). Journal of Economic Entomology 91(2): 410-418.

Nguyen AT, Newkirk AJW, Kitron UD, Chaves LF. 2014. Seasonal weater, nutrients, and conspecific presence impacts on the southern house mosquito oviposition dynamics in combined sewage overflows. Journal of Medical Entomology 49(6): 1328-1338.

Omolade OO, Adetutu SA. 2018. Oviposition and breeding water sites preferences of mosquitoes within Ojo Area, Lagos State, Nigeria. Biomedical Journal of Scientific and Technical Research, 7(5): 1-7.

Pailan S, Gupta D, Apte S, Krishnamurthi S, Saha P. 2015. Degradation of organophosphate insecticide by a novel *Bacillus aryabhattai* strain SanPS1, isolated from soil of argricultural field in Burdwan, West Bengal, India. International Biodeterioration & Biodegradation 103: 191-195.

doi:10.6342/NTU202001573

Patil CD, Patil SV, Salunke BK, Salunkhe RB. Prodigiosin produced by *Serratia marcescens* NMCC46 as a mosquito larvicidal agent against *Aedes aegypti* and *Anopheles stephensi.* Parasitology Research 109: 1179-1187.

Paula AR, Carolino AT, Paula CO, Samuels RI. 2011. The combination of the entomopathogenic fungus *Metarhizium anisopliae* with the insecticide Imidacloprid increases virulence against the dengue vector *Aedes aegypti* (Diptera: Culicidae). Parasites & Vectors 4: 8.

Perich MJ, Kardec A, Braga IA, Portal IF, Burge R, Zeichner BC, Brogdon WA, Wirtz RA. 2003. Field evaluation of a lethal ovitrap against dengue vectors in Brazil. Medical and Veterinary Entomology 17(2): 205-210.

Ponnusamy L, Böröczky K, Wesson DM, Schal C, Apperson CS. 2011. Bacteria stimulate hatching of yellow fever mosquito eggs. PLoS One 6(9): e24409.

Ponnusamy L, Wesson DM, Arellano C, Schal C, Apperson CS. 2010. Species composition of bacterial communities influences attraction of mosquitoes to experimental plant infusions. Microbial Ecology 59: 158-173.

Ponnusamy L, Xu N, Böröczky K, Wesson DM, Ayyash LA, Schal C, Apperson CS. 2010. Oviposition responses of the mosquitoes *Aedes aegypti* and *Aedes albopictus* to experimental plant infusions in laboratory bioessays. Journal of Chemical Ecology 36: 709-719.

doi:10.6342/NTU202001573

Ponnusamy L, Xu N, Nojima S, Wesson DM, Schal C, Apperson CS. 2008. Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by *Aedes aegypti*. Proceedings of the National Academy of Sciences 105(27): 9262-9267.

Ponnusamy L, Xu N, Stav G, Wesson DM, Schal C, Apperson AS. 2008. Diversity of bacterial communities in container habitats of mosquitoes. Microbial Ecology 56: 593-603.

Raghavendra K, Barik TK, Reddy BPN, Sharma P, Dash AP. 2011. Malaria vector control: from past to future. Parasitology Research 108: 757-779.

Rao DR, Mani TR, Radendran R, Joseph AS, Gajanana A, Reuben R. 1995. Development of a high level of resistance to *Bacillus sphaericus* in a field population of *Culex quinquefasciatus* from Kochi, India. Journal of the American Mosquito Control Association 11(1): 1-5.

Rosner B. 2011. Fundamentals of Biostatistics. 7th ed. Boston, MA: Brooks/Cole

Santos SRA, Santos MAVM, Regis L, Albuquerque CMR. 2003. Field evaluation of ovitraps consociated with grass infusion and *Bacillus thuringiensis* var. *israelensis* to determine oviposition rates of *Aedes aegypti*. Dengue Bulletin 27: 156-162.

doi:10.6342/NTU202001573

Seyoum A, Abate D. 1997. Larvicidal efficacy of *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* on *Anopheles arabiensis* in Ethiopia. World Journal of Microbiology & Biotechnology 13: 21-24.

Shelomi M. 2019. Bacterial and eukaryote microbiomes of mosquito habitats in dengue-endemic southern Taiwan. Journal of Asia-Pacific Entomology 22(2): 471-480.

Sota T, Mogi M, Hayamizu E. 1994. Habitat stability and the larval mosquito community in treeholes and other containers on a temperate island. Researches on Population Ecology 36: 94-104.

Sumba LA, Guda TO, Deng AL, Hassanali A. 2004. Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of mibrobial origin. International Journal of Tropical Insect Science 24(3): 260-265.

Sumba LA, Ogbunugafor CB, Deng AL, Hassanali A. 2008. Regulation of oviposition in *Anopheles gambiae s.s.*: role of inter- and intra-specific signals. Journal of Chemical Ecology 34: 1430-1436.

Tabashnik BE, Cushing NL, Finson N, Johnson MW. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). Journal of Economic Entomology 83(5): 1671-1676.

doi:10.6342/NTU202001573

Thomas MB, Read AF. 2007. Can fungal biopesticides control malaria? Nature Reviews Microbiology 5: 377-383.

Thorne L, Garduno F, Thompson T, Decker D, Zounes M, Wild M, Walfield AM, Pollock TJ. 1986. Structural similarity between the Lepidoptera- and Diptera-specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. "*kurstaki*" and "*israelensis*". Journal of Bacteriology 166(3): 801-811.

Wang XL, Yao YJ. 2011. Host insect species of *Ophiocordyseps sinensis*: a review. Zookeys 127: 43-59.

Wong J, Stoddard ST, Astete H, Morrison AC, Scott TW. 2011. Oviposition site selection by the dengue vector *Aedes aegypti* and its implications for dengue control. PLoS Negl Trop Dis. 5(4):e1015.

World Health Organization. 2005. Guidelines for laboratory and field testing of mosquito larvicides. Geneva, World Health Organization**:** 39

Xu Y, Orozco R, Wijeratne EMK, Artiles PE, Gunatilaka AAL, Stock SP, Molnar I. 2009. Biosynthesis of the cyclooligomer depsipeptide bassianolide, an insecticidal virulence factor of *Beauveria bassiana*. Fungal Genetics and Biology 46(5): 353-364.

doi:10.6342/NTU202001573

Yee DA, Kesavaraju B, Juliano SA. 2007. Direct and indirect effects of animal detritus on growth, survival, and mass of invasive container mosquito *Aedes albopictus* (Diptera: Culicidae). Journal of Medical Entomology 44(4): 580-588.

Yee DA, Kneitel JM, Juliano SA. 2010. Environmental correlates of abundances of mosquito species and stages in discarded vehicle tires. Journal of Medical Entomology 47(1): 53-62.

Yee DA, Allgood D, Kneitel JM, Kuehn KA. 2012. Constitutive differences between natural and artificial container mosquito habitats: vector communities, resources, microorganisms, and habitat parameters. Journal of Medical Entomology 49: 482-491.

Yuan Z, Zhang Y, Cai Q, Liu EY. 2000. High-level field resistance to Bacillus sphaericus C3-41 in Culex quinquefasciatus from southern China. Biocontrol Science and Technology 10(1): 41-49.

Zahiri NS, Su T, Mulla MS. 2002. Strategies for the management of resistance in mosquitoes to the mosquito control agent *Bacillus sphaericus*. Journal of Medical Entomology 39: 513-520.

Zahiri NS, Mulla MS. 2006. Ovipositional and ovicidal effects of the microbial agent *Bacillus thuringiensis israelensis* on *Culex quinquefasciatus* Say (Diptera: Culicidae). Journal of Vector Ecology 31(1): 29-34.

doi:10.6342/NTU202001573

Zimmermann G. 1993. The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. Pest Management Science 37(4): 375-379.

doi:10.6342/NTU202001573