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Aedes aegypti is the mosquito vector for dengue fever and has historically been considered to prefer ?clean? water for development. A 2006 study by the Centers for Disease Control and Prevention (CDC) demonstrated large numbers of adult Ae. aegypti mosquitoes emerging from septic tanks in Puerto Rico. The purposes of this study were (1) to definitively document larval presence in septic tanks and evaluate the water properties and environmental factors related to that presence, (2) examine the use of 2,2-dichlorovinyl dimethyl phosphate impregnated strips for control of mosquito productivity in septic tanks, and (3) use Geographic Information Systems to examine the association between the presence of septic tanks and other environmental factors and the incidence of dengue in Puerto Rico.

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Abstract

Evaluation of *Aedes aegypti* Presence and Abundance in Septic Tanks and Their Impacts on Dengue Transmission

Ronald L. Burke, Doctor of Public Health, 2009

Thesis directed by: David M. Claborn
Assistant Professor
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Background: *Aedes aegypti* is the mosquito vector for dengue fever and has historically been considered to prefer 'clean' water for development. A 2006 study by the Centers for Disease Control and Prevention (CDC) demonstrated large numbers of adult *Ae. aegypti* mosquitoes emerging from septic tanks in Puerto Rico. The purposes of this study were (1) to definitively document larval presence in septic tanks and evaluate the water properties and environmental factors related to that presence, (2) examine the use of 2,2-dichlorovinyl dimethyl phosphate impregnated strips for control of mosquito productivity in septic tanks, and (3) use Geographic Information Systems to examine the association between the presence of septic tanks and other environmental factors and the incidence of dengue in Puerto Rico.

Materials and Methods: A miniaturized funnel trap (Vietrap) was used to sample 89 septic tanks in the Puerto Rican community of Playa/Playita. Water quality samples were also obtained from each septic tank. Adult emergence trapping was conducted on 25 septic tanks in the community of Las Mareas in order to test the effectiveness of the impregnated strips for controlling mosquito productivity. Maps of sewerage lines were used to identify populated areas which did not have a sewerage system (i.e. used septic tanks) and compared to laboratory confirmed cases of dengue from 2003 - 2008, as reported by the CDC.

Results: Larvae were recovered from 18% of the sampled tanks. Larval presence was positively associated with uncapped tank access ports and cracked walls. Larval abundance was positively associated with uncapped tank access ports, cracked walls, and surface area, and was negatively associated with total dissolved solids. Adult emergence was significantly reduced after one month in treated (median 6) versus untreated (median 40) septic tanks. A significant association between the absence of sewerage and dengue was noted during the rainy season, but it did not explain the variation in the incidence of disease.

Conclusion: This study provides evidence that *Ae. aegypti* larvae are present in septic tanks and that septic tanks should be considered when developing mosquito control strategies, especially in areas where dengue or yellow fever are endemic.

EVALUATION OF *AEDES AEGYPTI* PRESENCE AND ABUNDANCE IN SEPTIC TANKS AND THEIR IMPACTS ON DENGUE TRANSMISSION

by

Ronald L. Burke

Thesis submitted to the Faculty of the
Department of Preventive Medicine and Biometrics of the
Uniformed Services University of the Health Sciences
in partial fulfillment of the
requirements for the degree of
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Preface

This study would not have been possible without the assistance and mentorship of my thesis advisor, Dr. David Claborn. Nor would it have been possible without the tutelage, guidance, and especially patience of my committee members: Dr. Roberto Barrera, Dr. John Grieco, Dr. Timothy A. Kluchinsky Jr., Dr. Michael Lewis, and Dr. Edward Mitre. I also wish to acknowledge all of my instructors and faculty at the Uniformed Services University for helping to educate and prepare me for this endeavor, and especially Dr. Cara Olsen for her assistance with the statistical analysis and Penny Masuoka for her assistance with the GIS analysis. I also wish to thank my favorite sister-in-law in South Carolina, Allison Burke, for her assistance with proofing and editing this 'short' paper.

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Dedication

This thesis is dedicated to my loving wife, Kristina. Knowing you has made this whole experience worthwhile.

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Chapter 1 – Introduction and Background

Disease Background

Dengue is caused by any one of four antigenically-distinct serotypes of the dengue virus in the *Flaviviridae* family and is transmitted by mosquitoes.[1] The disease is found in over 100 countries throughout Africa, the Americas, Southeast Asia, and the Western Pacific. Over 2.5 billion persons are currently living in endemic regions, or regions where dengue transmission is rare, but still possible.[2] Dengue has four clinical manifestations: 1) undifferentiated illness, 2) classic dengue fever, 3) dengue hemorrhagic fever, and 4) dengue shock syndrome.[1] Undifferentiated illness is the most common, and infected individuals are usually asymptomatic or mildly symptomatic. Classic dengue fever is characterized by fever, retro-orbital pain, myalgia, and arthralgia. Muscle and joint pain are often severe, resulting in the disease's common name of "break-bone" fever. [1,3,4] Dengue hemorrhagic fever and dengue shock syndrome are progressively more severe forms of the disease in which vascular permeability is compromised, leading to blood loss, shock, cardiovascular failure and possibly death. Fatality rates in misdiagnosed or mismanaged cases can exceed 20%; however, with correct management and proper fluid replacement, the case fatality rates for dengue hemorrhagic fever may be as low as 1-2%.

The first reported outbreak of dengue fever in North America, Africa, and Asia occurred between 1779 and 1780.[5] The disease normally occurred in persons with a history of visiting the tropics and was generally a mild, non-fatal disease. Major epidemics were typically separated by long intervals of 10-14 years because susceptible populations were only exposed to new serotypes if both the mosquito vector and the virus were able to survive the long voyage by sail between population centers. Shortly after World War II (WWII), a dengue epidemic was reported in Southeast Asia and subsequently spread as a pandemic throughout the tropics and subtropics. Increased global travel and decreased travel times led to shortened periods between epidemics and the presence of multiple serotypes (hyperendemicity) in the same geographic area. This in turn resulted in the eventual emergence of dengue hemorrhagic fever and dengue shock syndrome in Southeast Asia during the 1950s.

The Rise of Dengue in the New World

In 1947 the Pan American Health Organization (PAHO) adopted CD1.R1, a resolution to eradicate the *Aedes aegypti* (Linnaeus) mosquito from the western hemisphere.[6] Although elimination of yellow fever was the council's primary intent, the program also targeted dengue fever as the *Ae. aegypti* mosquito was the primary vector for both diseases.[7] Under the massive eradication program, vector control teams conducted house-to-house operations to eliminate the mosquito through insecticidal fogging, application of residual insecticides like dichlorodiphenyltrichloroethane (DDT), and removal of breeding sites. By the late 1960s, *Ae. aegypti* was eradicated from all but 4 of the 27 American countries.[6,8] The four countries that failed to eradicate the insect were Guyana, Suriname, the United States, and Venezuela. Efforts in the Caribbean were less successful though, as only 2 of 26 nations and territories successfully eradicated the vector. In 1973 dengue replaced yellow fever as the driving force behind the eradication program. Throughout the next several decades, PAHO continued to issue resolutions which reaffirmed its intention to eradicate the vector and urged governments to continue working on research in vector-borne diseases and control. Unfortunately during the late 1960s and early 1970s, the program began to experience some setbacks, and near the end of the 20th century, the mosquito had re-infested all of the countries from which it had been previously eradicated.[9] (Figure 1).

There are several factors which contributed to the eradication program's ultimate failure. Although the program eliminated *Ae. aegypti* from 85% of the countries within the Western Hemisphere, re-introduction from neighboring countries which had failed to eradicate the vector was a persistent problem. For instance, Panama successfully eradicated the *Ae. aegypti* mosquito on five separate occasions.[6] Each success was followed by a re-infestation a few years later, the last occurring in 1985 and continuing through the present day. Even if PAHO had successfully eliminated the vector from the entire Western Hemisphere, re-infestation would likely still have occurred due to increased global travel, especially by air, from countries where the vector was still present.[10]

Another factor which contributed to the program's failure was the switch from DDT to other residual insecticides and eventually to ultra-low volume (ULV) space sprays.[11] Unfortunately, these new chemicals were often noticeably more expensive than DDT and had their own risks of causing harm. Many insects developed resistance to these chemicals as well.[12-15] A further downside of the recent switch to ULV spraying is the lack of residual or repellent activity by the chemicals. Thus, they do little to decrease the overall number of mosquitoes and the burden of disease.[9,11]

Urban growth also played a role in the program's failure.[16] The construction of new homes meant more breeding sites for the mosquitoes, and subsequently insufficiency resources for vector control. Governments were unable to keep pace with the rapid urbanization and new neighborhoods often had inadequate water, sewerage, and other public services. A study in Venezuela found a correlation between the number of containers with *Ae. aegypti* larvae and the frequency of interruptions to the water supply.[17] This problem was further compounded by the public's incomplete understanding regarding the importance of *Ae. aegypti*. Many of the individuals surveyed, reported the larvae had been present for some time, and did not understand their importance as a public health hazard or the need to remove potential larval habitats.

An additional factor which contributed to the eradication program's failure was the rising cost of vector control operations. The door-to-door source-reduction campaigns were highly effective but they were also quite expensive. Faced with the continued re-infestations from neighboring countries, governments began to question the value of spending so much money on what appeared to be an unrealistic goal. Dengue control programs were scaled back and eventually merged with malaria control and other similar programs.[11] Fewer control teams were sent into the field and more emphasis was placed on community participation. Unfortunately, the communities often failed to recognize the importance of *Ae. aegypti.*[18] Even when communities were aware of *Ae. aegypti's* public health importance, common misconceptions about larval habitats, personal responsibility, and insecticide use thwarted attempts to control or reduce the mosquito population.

The failure of the Pan American Health Organization's eradication program allowed *Ae. aegypti* to reclaim its previous domain and inhabit new areas as well.[19] Increased numbers of global travelers resulted in frequent importations of new serotypes into the region. Some countries now have all four serotypes present. The combined increase in competent vectors and new serotypes led to a resurgence of dengue in the Americas. In 1980, there were 66,011 cases of dengue. By 2000, the annual number of new cases exceeded 700,000 and continues to grow.[19] The same factors which contributed to the program's failure in the past are still at work today and continue to challenge public health officials.

Dengue in Puerto Rico

The first recorded outbreak of dengue fever in Puerto Rico occurred in 1914.[20] Since then, the territory has experienced at least ten subsequent epidemics, the last occurring in 2007 – 2008.[21-24] The Centers for Disease Control and Prevention (CDC) established a laboratory based surveillance system in 1975 to track dengue hemorrhagic fever and dengue shock syndrome. The system is primarily passive in nature and relies on case reports and serum submission from local physicians to the CDC laboratory in San Juan. An active based surveillance program for dengue hemorrhagic fever was added in 1988 to cover in-patient hospital admissions; however, the system has been estimated to underreport the true incidence by a factor of at least 2.85.[25,26] Between 1975 and 1985, 47,196 suspected cases of dengue fever were reported.[27] Nineteen percent of the 8,816 adequate laboratory samples during this time period were confirmed by CDC's laboratories. Three percent (230) of persons with a laboratory confirmed diagnosis were hospitalized. By 2008, the total number of reported cases was 149,673, with 46,782 of them receiving laboratory confirmation (CDC, unpublished data). During this period, 206 dengue related deaths were also reported.

Immediately prior to the 2007 dengue outbreak, the only dengue serotypes in Puerto Rico were type 2 and 3; however, all four serotypes are now present on the island.[24] Between January and October 2007, a total of 7,824 suspected cases of dengue fever were reported to Puerto Rican health authorities. Of these, 2,301 were laboratory confirmed. While dengue transmission on the island occurs throughout the year, peak transmission is generally during the latter half of the year which corresponds with the rainy season.[28,29] This pattern is especially evident in the southeast portion of the island which has a noticeable dry season.

Factors Affecting Disease Transmission and Severity

An infection with one serotype of dengue will provide life-long immunity against infections from the same serotype.[5,30,31] Dengue transmission threshold levels are therefore affected by a population's immune status (herd immunity).[32] As the number of immune individuals within the community increases, a level will eventually be reached where continued transmission is no longer possible under the current situation.

Unfortunately, life-long immunity is serotype specific, and there is only a transient immunity against the other serotypes. The cross-reactivity among serotypes is also thought to be responsible for dengue hemorrhagic fever and dengue shock syndrome through a process known as immune enhancement.[30,33-35] In immune enhancement, non-neutralizing antibodies from one viral serotype enhance the uptake of other serotypes by mononuclear phagocytes. However, once inside the phagocyte the virus is not killed. Instead, it replicates and results in a more severe form of the disease which is often seen in secondary dengue infections.

Individual genetics and ethnicity can affect disease severity, but not infection rates. A study in Cuba determined that dengue infection rates during the 1981 DEN-2 outbreak were equal in blacks and whites.[36,37] However, a retrospective analysis of hospitalizations during the outbreak indicated whites where significantly more likely to have the severe form of the disease, and they also had higher case fatality rates. These findings were consistent with a previous study which showed a difference in the incidence of dengue hemorrhagic fever among blacks and whites.[38] While the reason for this has not been determined, one hypothesis is blacks may possess a gene which provides protection against developing dengue hemorrhagic fever. This hypothesis is supported by a study in Thailand which demonstrated varying resistance to severe secondary dengue infections in relation to an individual's human leukocyte antigen (HLA) allele.[39]

Age also appears to have a role in determining disease severity among infected individuals.[30,31,40] During initial dengue infections, young children will often have inapparent or mild symptoms while adults typically experience classical dengue fever. However, when infected with a second serotype, greater age appears to be protective, as the youngest children typically are at highest risk for developing increased vascular permeability and dengue hemorrhagic fever.[41] In infants, passive transfer of immunity may increase the risk of developing a severe infection.[42,43] During pregnancy, IgG dengue antibodies can be transferred from the mother to the fetus. A subsequent infection with a new serotype will result in immune enhancement and dengue hemorrhagic fever in the infant.

Viral strain differences may also affect disease pathogenicity and virulence as researchers have observed variable effects according to the virus genotype.[30,31] Genetic differences between an American genotype and two Southeast Asian genotypes are believed to be responsible for the recent increased virulence and development of dengue hemorrhagic fever from secondary infections with dengue type 2 virus.[44] The new Asian genotypes produce higher viral outputs, which may increase their ability to infect more mosquitoes than the American genotype.[45] The apparent advantage in replication and infection could lead to the Southeast Asian genotypes eventually replacing the American genotype in the Western hemisphere.

The level of viremia which is needed in humans to naturally infect *Ae. aegypti* is unknown, but is generally considered to be high.[46] Laboratory studies suggest mosquito susceptibility is dependent on geographic location and whether the virus is sylvatic or endemic in nature.[4] Vertical transmission of the dengue virus is possible, but is relatively low in most species of *Aedes* mosquitoes.[47] An exception is the *Aedes mediovittatus* (Coquillett) mosquito which has high rates of filial infection, and may be a reservoir for dengue during inter-epidemic periods.[48,49]

Ambient temperature affects dengue transmission through two ways.[50,51] First, warmer temperatures generally result in shorter vector development times. Second, warmer temperatures also lead to shorter extrinsic incubation periods. The extrinsic incubation period is the time between when the mosquito feeds on an infected individual and the time when the mosquito becomes infective and can transmit the disease to a susceptible individual. In general, the extrinsic incubation period is between 10 and 14 days, with warmer climates being more favorable for dengue transmission than their cooler counterparts.[31,50]

In Puerto Rico, several other factors were also associated with dengue transmission and antibody prevalence.[52] Environmental risk factors associated with an increased prevalence of dengue included neighborhood tree heights of at least 20 feet, increased shade, urbanization, and the presence of domestic animals. There was also an inverse relationship between dengue transmission and socioeconomic status. Screening of windows and doors was associated with a lower incidence of dengue transmission. Clustering of cases within households was also observed. The clustering was most likely a result of frequent interrupted feedings by *Ae. aegypti*, a behavior which has been observed in the species.

Vector Biology

Dengue is primarily transmitted by the mosquito species *Ae. aegypti* and occasionally by *Aedes albopictus* (Skuse) and other *Aedes spp.* mosquitoes.[53] *Aedes aegypti*'s role as the primary vector for dengue is largely due to the mosquito’s close affinity with man. The insect is generally a day-biter, with activity peaks in mid-morning and again in the late afternoon.[53] Females are highly anthropophilic (>95%) and will feed multiple times during a single gonotrophic cycle.[54,55] *Ae. aegypti* adult resting sites are usually dark, sheltered locations within homes such as closets, bathrooms, ceiling corners, and under furniture. The average adult lifespan for *Ae. aegypti* is three to six days for males and eight to fifteen days for females.[53] Flight distances are often less than 50 m, and rarely over 100 m due to the abundance of food, mates, and oviposition sites within human settlements.[56,57] However, at least one study has demonstrated that the *Ae. aegypti* will fly up to 320 m when there are few suitable oviposition sites. This finding suggests that current spray control methods that only focus on a 50 – 100 m area may be ineffective.[58]

Aedes aegypti eggs are highly resistant to desiccation and large numbers of eggs can survive for several months.[46] The resistance to desiccation allows the mosquito to survive prolonged droughts and poses problems for control programs. The eggs are laid above the water line and only hatch once water levels rise. The delayed hatching ensures water will be present for the entire metamorphosis.

Aquatic Habitat Factors in Relation to Oviposition and Larval Development

Aedes aegypti oviposition sites are typically in artificial, man-made containers such as flower vases, water storage drums or tanks, and discarded plastic or metal containers.[53,59] The surface area and volume can influence the suitability of a container for oviposition as adult females have been shown to lay more eggs in larger and deeper containers.[60] The mosquitoes are also attracted by certain visual factors and prefer containers which have solid, dark colors and low reflectance.[61] Once at a container, the presence of *Ae. aegypti* or other mosquito larvae can discourage the mosquito from ovipositing.[62] Conversely, the presence of the predaceous species *Mesocyclops longisetus*, a copepod, will stimulate oviposition, possibly through chemical mediators like 3-carene or α-terpinene.[63,64] When larval habitats are abundant (i.e. during the rainy season), females often exhibit "skip oviposition" whereby they will bypass undesirable sites and lay their eggs in multiple sites.[65] Under less favorable conditions, the mosquito can lay its eggs in only a few locations or sometimes all at once.

An artificial container's construction material may play a role in determining the suitability of the aquatic environment. The presence of certain metal liners (e.g. copper and bronze) in artificial containers has been associated with decreased *Ae. albopictus* and *Ae. aegypti* larval populations.[66,67] Shading of the container may also have an impact on the water temperature which affects the suitability of the aquatic habitat and larval development rates.[68-70]

Larval development and survival is determined by temperature and the availability of food resources.[71] Larvae which are initially well fed during the 1st and 2nd instar stages are generally more resistant to subsequent starvation and develop into larger adults than larvae from food scarce environments.[72] The type of food present may also influence larval survival as *Ae. aegypti* larvae were found to have higher survival and development rates after feeding on non-natural food sources, while *Ae. albopictus* larvae had better rates with natural (i.e. leaf debris) food sources.[73] While the time necessary for complete development can be as short as 7.2 days at 35 ºC, larger larval survival rates (89 – 93%) are usually between 20 – 30 ºC, where development takes 8.4 – 13.7 days.[68] At temperatures below 10 ºC, or above 40 ºC, all larvae have been noted to die before completing development. However, prolonged exposure of larvae to low temperatures can improve survival rates in subsequent generations.[74] At least in Australia, the availability of food is more important than temperature for determining larval development rates as larvae generally developed five days faster in shaded containers under trees with plentiful detritus (organic debris) than in containers which were in the open and 2.6 ºC warmer.[68]

Studies have found that in addition to temperature and the availability of food, several other physicochemical water properties of aquatic habitats can influence immature development and survival of mosquitoes. Investigators in Kenya noted a positive association between *Culex quinquefasciatus* (Say) larval abundance and turbidity and a negative association with dissolved oxygen.[75] Another study in Nigeria found a relationship between *Culex ingrami* (Edwards) larval abundance and the depth, surface area, total dissolved solids (TDS), and conductivity of the water. The optimal pH for minimal larval developmental time and maximal growth rate is a neutral 7.0; however, the *Ae. aegypti* larvae can develop between the ranges of 4.0 and 11.0.[76]

Historically, *Ae. aegypti* larvae have been observed in relatively unpolluted surface waters, but several studies suggest the species is developing in other cryptic habitats like sewers, subterranean cisterns, and septic tanks.[77-79] In Malaysia, *Ae. aegypti* larvae were recovered from the effluent portion of septic tanks, but not from the portions of the septic tanks containing raw sewage.[80] Another study reported finding *Ae. aegypti* larvae in

Indian septic tanks, but did not specify the activity status of the tanks, the type of septic tank, or whether the larvae where found in the effluent tank or the holding/settling tank.[81]

These subterranean sites are important for several reasons. First, they can produce large numbers of adult mosquitoes.[82] Second, proximity to subterranean larval habitats is positively associated with dengue seropositivity.[83] Third, and perhaps most important, human access to subterranean habitats is often limited. This limitation renders treatment with larvicides more difficult, necessitating the development of new control measures in the future.

Several studies have determined that in some instances only a few container types (<40%) may be responsible for more than 80% of the pupae, and presumably the adult population.[84,85] These "super-producing" containers vary by location. For instance, in cemeteries, flower pots and vases are important sources of mosquito production due to their relative abundance and infrequent cleanings.[86,87] However, in homes where the water is frequently changed, it is unlikely that mosquito pupae will have sufficient time to develop, and the vases and flower pots are thus less important sources of adult mosquitoes. On the other-hand, septic tanks are generally serviced annually, or not at all. If *Ae. aegypti* larvae are present in septic tanks containing raw sewage, the infrequent service would provide sufficient time for development, which could lead to large numbers of adult mosquitoes.

Mosquito Sampling Techniques and Applicability

One of the most common methods for sampling *Ae. aegypti* is the use of ovitraps.[88,89] Briefly, the trap consists of a black, one pint, glass container, a wooden or fiberboard paddle, and approximately 100 mL of water.[90,91] The use of hay infusions has also been shown to improve trap performance. The paddle is positioned just above the water where gravid females can oviposit. Unfortunately, while ovitraps are both sensitive and cost-effective for determining the presence or absence of *Ae. aegypti* in a given area, their results are not predictive of the actual number of adult mosquitoes or the burden of disease.[59,92]

Other methods for trapping adult mosquitoes include the use of sticky traps, back-pack aspirators, landing collections and visual/chemical traps such as the CDC light trap and the BG-Sentinel Trap (Biogents AG; Regensburg, Germany).[93-95] Chemical attractants like carbon dioxide and octenol are often used with visual traps in order to improve their performance.[96] However, similar to ovitraps, the data collected using the above methods cannot be used for dengue risk assessments.[59] One reason for this is that these methods only collect a fraction of the total number of mosquitoes so that it is difficult to determine the actual number of adult mosquitoes. This problem is further compounded by the fact that the relationship between adult mosquitoes per person and the burden of disease is not as clearly defined as is the relationship between pupae per person and the burden of disease.[97]

Despite these limitations, adult traps are still useful in the field for various research purposes. Recently, the CDC used adult emergence traps to sample septic tanks in the Puerto Rico community of Playa/Playita.[82] The emergence traps were constructed from

3.8L (1 gallon) plastic jugs which had a screened opening on one end and a fabric sleeve over a hole in the middle of the container. The fabric sleeve was placed over the end of the septic tank vent pipe or over the tip of a large inverted funnel which was placed over the septic tank opening. Cracks in the tank walls were then sealed with Great Stuff™ foam sealant (Dow Chemical Company; Midland, Michigan) in order to prevent the mosquitoes inside the septic tanks from leaving the tanks through alternative exits and inhibit new mosquitoes from entering the tanks. The traps were sampled for four consecutive days. A total of 135 septic tanks were sampled and adult *Ae. aegypti* were collected in 48 tanks. The mean daily emergence number from open septic tanks was 44 ± 62 (95% Confidence Interval), but one tank produced over 1,440 per day. Statistical analysis indicated open/broken tanks and shorter vent pipes were associated with larger numbers of emerging adults. The estimated daily adult emergence total from septic tanks for the community of Playa/Playita was 4.4 *Ae. aegypti* per person. Assuming these adults were all newly emerged, and the average pupal stage duration was approximately two days, this would have equated to a standing pupal count of eight to nine pupae per person. Although this level of pupae is theoretically sufficient for dengue transmission, no cases of dengue were reported in Playa/Playita during the study (November 2005 – January 2006) and the researchers did not detect dengue virus in any of the 2,212 sampled adult females.

In order to provide evidence that the mosquitoes from the CDC study in Playa/Playita were newly emerged, and not simply resting adults, it is necessary to sample the septic tank waters for mosquito larvae. Sampling of surface containers can be accomplished through sieving, sweep nets, dipping, and pipettes.[98-101] In subterranean sites like wells and septic tanks, these methods are often difficult to use and sampling is generally passively conducted using funnel traps.[102-106] The basic trap is constructed using a funnel, a counterweight, and a jar, bottle, or similar structure for the reservoir. The reservoir is fitted over the funnel tip and the trap is filled with water to approximately the three-quarters full level, ensuring there is a small air gap in the reservoir container. Upon entering the well water, the counterweight inverts the trap so that the mouth of the funnel is facing downwards. As the mosquito larvae forage for detritus, they will move throughout the water column and some larvae will move under the mouth of the funnel and subsequently enter the trap reservoir when they surface to breathe. The smaller diameter of the funnel tip opening, as compared to the reservoir diameter, reduces the likelihood of the larva exiting the trap as it continues to forage. Upon removal from the well water, the trap flips ‘right-side’ and any larvae in the reservoir are trapped for examination. Advantages of the funnel trap include its simple construction, relatively low cost, and ease of use.

Funnel trap performance is affected by population density and well diameter.[106] At low population densities, or in large wells, the trap may be incapable of detecting larval presence. However, using known trap results from known population densities and well diameters, it is possible to develop regression lines for predicting the total larval populations based on trap results in the field. The trap draft will also influence performance with shallower models generally having higher trap counts than their deeper peers.

The species of mosquito larvae present in the well will also affect trap performance. Larvae with greater vertical movements like *Ae. aegypti* are more likely to become trapped in the reservoir than larvae that remain at or near the water's surface like *Cx. quinquefasciatus*.[99,104] The duration of trapping will affect trap performance with trap durations of 16 or 24 hours collecting significantly more larvae than trap durations of only 8 hours.[105] Funnel traps may also perform better with 3rd and 4th instar larvae than with younger stages.[106] Due to the limited diving activity of pupae, funnel traps are less effective for sampling this stage than they are for larvae, so a floating square with parallel v-shaped troughs was developed for pupal samples.[59] However, this trap cannot be employed passively as it requires repeated insertion into the well water by the investigator in order to disturb the water's surface and stimulate pupae diving behavior. Two final important factors to consider when determining whether to use a funnel trap are the opening diameter of the well compared to the funnel mouth as the former must be greater than the latter, and the potential for the funnel mouth opening to become obstructed with floating debris.

Early larval sample results were reported using the House, Container, and Breteau Indices.[107,108] The House Index is the percentage of houses infested with larvae or pupae, while the Container Index is the percentage of water-filled containers which are infested. The Breteau Index is the most recent of the three and is the number of positive containers per 100 households in an area. These larval (*Stegomyia*) indices were used to measure mosquito control efforts during early 20th century Yellow Fever eradication programs; an example being in South and Central America where a Container Index of less than 10 was used to designate ‘safety zones’.[108] More recently the Breteau Index was used to identify geographic areas near Havana, Cuba which had a high risk of dengue transmission.[109] Although several other studies also found associations between the indices and the prevalence of dengue or yellow fever, their overall usefulness is limited.[59,110] The observed associations between larval indices and disease were regional, and ‘safe’ levels in one area were not necessarily safe in another area. Moreover, the larval indices failed to account for the immune status within the local population, the effect of temperature variations, differences in productivity between various containers, and ratio of immatures to people.

In lieu of these shortcomings, a pupal index was developed. *Aedes aegypti* pupae are readily identified and counted within a community, and have a relatively low mortality rate, which makes them ideal for modeling.[71,111,112] Using temperature and assumed seroprevalence values for the local population (herd immunity), it is possible to determine the theoretical number of pupae per person necessary for a dengue epidemic to occur after the introduction of an infected individual into the community (**Table 1**).[32,71,113] As temperature increases, or herd immunity decreases, the number of pupae per person required to support a dengue outbreak decreases. Assuming the average winter temperature in the southeast portion of Puerto Rico is at least 24 ºC and herd immunity is 33%, 4.47 pupae per person are needed for a dengue outbreak.[114] During the warmer summer months when temperatures are at least 26 ºC, only 2.03 pupae per person would be necessary for an outbreak to occur.

Prevention of Dengue

Due to the previously mentioned problems with serotype cross-reactivity and immune enhancement there are currently no licensed vaccines for dengue fever, although several possibilities are in various stages of development and testing.[115,116] Treatment for dengue is largely symptomatic, so prevention efforts are focused on controlling the mosquito vector. Control efforts can be directed at either the adult or immature stages.

Examples of adult control methods include the use of residual insecticides, aerosols, lethal ovitraps, genetically modified mosquitoes, and personal protective measures.[117] Although effective residual insecticide treatments may last for months, they are extremely labor intensive, and therefore used infrequently. Aerosols (e.g. ULV sprays) are quite effective against caged sentinel mosquitoes, but aside from some initial studies in Thailand, they appear to have little effect on wild mosquito populations (CDC, unpublished data).[117-119] A likely explanation for their limited effectiveness is the tendency for female *Ae. aegypti* adults to rest in secluded areas where they are unlikely to come in contact with the insecticide while it is aerosolized and suspended. In addition to the limited effect of aerosols on adult mosquito populations, there is also no evidence that their use reduces the burden of diseases during epidemics.[7] Despite these limitations, ULV application is still recommended by the Pan American Health Organization during dengue outbreaks as "any control method that reduces the number of infective mosquito adults, even for a short period of time, should reduce virus transmission during that time."[7] ULV applications also have the added benefit of being readily visible, thereby helping to assure the local populace that the government is acting to address the problem.

Although the ovitrap was originally developed as a surveillance tool, it has since been modified to control adult mosquitoes as well.[120] A lethal ovitrap can be made by adding an insecticide (e.g deltamethrin) treated strip to the container or by using a adhesive tape in lieu of the ovistrip.[121] Sticky traps have the added benefit of being able to measure the actual mosquito visitation of traps rather than just mosquito oviposition. Lethal ovitraps have been shown to reduce the number of number of water filled containers with *Ae. aegypti* larvae and pupae, as well as the number of adults.[120] However, studies have indicated they are incapable of eliminating the species from the environment alone; most likely a result of oviposition in other containers within the area. Trap performance is also affected by placement height, with ground level traps performing better, and shelter from the wind, with leeward containers trapping more adults during the dry season.[121]A particular concern regarding the use of lethal ovitraps is the potential for non-lethality once the insecticide or adhesive strip deteriorates. Records of lethal ovitraps locations must be maintained so that the traps can be collected or the strips replaced, which requires time and manpower. Although biodegradable lethal ovitraps have been developed as a potential solution to this problem, they have not yet been perfected and questions still remain as to their suitability.[122]

Advances in genetics have increased interest in using genetically modified mosquitoes to reduce dengue transmission. Genetic modification seeks to accomplish one of two goals, population suppression or population replacement.[123] In population suppression, genetic modifications are made to the insect which will reduce, and hopefully eradicate, the species. Perhaps the most well known example of this is sterilization of male screwworm flies which leads to ineffectual mating and eventual elimination of the species from a geographic area. Unfortunately, methods of sterilization are often difficult and what may work for one species (e.g. the screwworm) may not work for another (e.g. the mosquito). An alternate to sterilization is the release of insects carrying a dominant lethal gene (RIDL). An example is the genetic modification of male *Ae. aegypti* mosquitoes so that they will not survive unless fed tetracycline.[124] During mating, the males will pass this gene on to their offspring which could eventually lead to species eradication. The alternative to population suppression is population replacement. In population replacement, the goal is not species eradication, but replacement with a modified species which is incapable of transmitting disease. An example is the genetic modification of *Ae. aegypti* to stimulate development of ribonucleic acid interference (RNAi) which provides resistance against dengue type 2 virus infection.[125] In addition to the technical difficulties, the widespread use of genetically modified mosquitoes is also limited by concerns over unintended consequences like effect on non-target species or improved transmission of other non-target diseases.[123]

The final aspect of adult mosquito control is the use of personal protective behaviors like screening, repellents, and permethrin treated clothing. While these methods do not control the number of adult mosquitoes, they do help to prevent disease transmission by reducing human exposure to infective mosquitoes.

Due to the above limitations of adult control, successful vector management programs often involve larval/pupal control programs as well. Control of mosquito immatures is primarily accomplished through source reduction and the use of larvicides and biologics. Source reduction efforts attempt to remove potential larval habitats from the environment in order to reduce mosquito productivity. When conducting source reduction, it is important to realize that the most abundant container may not necessarily be the most prolific source of pupae and adult mosquitoes.[84,85,112] An initial survey of the community may identify the significant sources of pupae and help direct subsequent reduction efforts.[59]

A final point to consider when designing source reduction programs is the issue of bottom-up versus top-down.[9] It has been suggested that programs which are ‘bottom-up’, or community-driven, are more likely to be sustained over the long-term than government directed or mandated programs, and are thus more likely to be successful in controlling mosquito development. Unfortunately, while a bottom-up program is regarded as more cost-effective, and therefore sustainable, than a top-down approach, it is slower to implement as it must rely on behavioral modification of the populace for success.[9] While the CDC’s community program in Puerto Rico has substantially increased public awareness about dengue, it has yet to change public behavior enough to reduce the mosquito burden to levels which do not support epidemic transmission.

When it is not possible to physically remove the container sources from the environment (e.g. septic tanks, wells), the use of insecticides like temephos can prevent mosquito development for several months.[126] Other potential methods for controlling larval development include using predaceous fish and copepods which feed on the larvae, the *Bti* protein from *Bacillus thuringiensis* serotype H-14 which is toxic to mosquitoes, or polystyrene beads which interfere with larval respiration.[127-129]

Insect growth regulators are another method for controlling larval populations.[130-133] Insect growth regulators generally work by inhibition of chitin synthesis (e.g. diflubenzuron) or mimicry of insect juvenile hormones (e.g. pyriproxyfen and methoprene). Chitin inhibitors lead to retention of exuviae and subsequent death following ecdysis. Juvenile hormone analogs disrupt the normal metamorphosis pathways and prevent complete development of the immature mosquito. Of particular benefit when using pyriproxyfen is the ability to use controlled release formulations for controlling larval populations over extended periods of time. Using resin coated strands of pyriproxyfen, investigators in Cambodia were able to significantly reduce adult emergence by 90% for 20 weeks and by at least 80% for 34 weeks.[132] Other benefits of insect growth regulators include approval for use in drinking water, limited to no activity against non-target species, and no residual taste or discoloration of the water.[134]

An added benefit of immature control, as compared to adult control programs, is the measurable effect on dengue transmission. Ultra-low-volume sprays may temporarily lessen the number of biting mosquitoes (all species), but as previously mentioned, they have not been shown to reduce the burden of disease. In contrast, pupal survey results and source reduction can be used to reduce the number of pupae per person in an area to levels which are unlikely to support dengue outbreaks.[32]

The Use of Geographic Information Systems in Dengue Surveillance

Geographic Information Systems (GIS) are useful for modeling vector borne disease incidence, and with the proliferation of free spatial data sources like Google Earth™

(Google; Mountain View, CA), they can be a cost-effective tool for countries with limited budgets.[135,136] GIS software has been used to temporally and spatially visualize dengue outbreaks, conduct vector surveillance programs, implement control measures, and perform cost-analysis assessments.

In Argentina, India, and Puerto Rico, GIS was used to identify dengue points of introduction within a community and analyze the subsequent spread of disease.[137-139] Spatial and temporal clustering of cases was noted in the studies, findings which are consistent with feeding behavior of *Ae. aegypti* (e.g. multiple blood-meals) and the extrinsic incubation period of the dengue virus.[50,54] The Puerto Rico analysis also noted a rapid spread of the disease within the community. This finding was important because countries like Argentina often use ULV around suspected cases of dengue to help prevent outbreaks. However, the rapid progression of the disease suggests this method may not be effective, and efforts should be directed at the entire community instead of focusing on the immediate vicinity of suspected or confirmed cases.

Several studies in Thailand have used GIS for vector surveillance.[140,141] In one study, dengue-infected *Aedes spp.* adults were detected with reverse transcriptase – polymerase chain reaction, enzyme linked immunosorbent assays, and other rapid diagnostics. Global positioning systems and GIS software were then used to identify spatial distribution and possible clustering for use as a dengue early warning system. In another study, GIS software was used to determine immature density and clustering within a community as a means of targeting control efforts. Vector surveillance was taken a step further in Argentina where GIS was used to develop models for predicting House and Breteau Indices for *Ae. aegypti* using satellite imagery and weather data.[142] The study found that Normalized Difference Vegetation Index (used to estimate vegetation health and coverage), precipitation, temperature, and humidity were predictive for both indices. Finally, it was through the use of GIS to examine mosquito densities in Playa/Playita that the relationship between septic tanks and *Ae. aegypti* was first noticed in the community.[82] After an intervention aimed at reducing the most significant surface containers for pupal productivity failed to reduce the number of adult mosquitoes which were captured from households, the investigators used GIS software to check for clustering of adults. Maps and aerial photographs of the community were digitized and GIS software was used to identify hot-spots and significant clustering of captured adult mosquitoes. A subsequent survey and adult emergence sampling revealed large numbers of mosquitoes were emerging from septic tanks, the first such reported occurrence in the Caribbean.

GIS mapping of dengue incidence and vector densities can also be useful for dengue control programs. GIS software was used in Thailand to identify dengue foci and *Aedes* positive containers within a village in order to direct vector control efforts.[143] Control efforts included container source reduction, screening of water jars, the use of *Mesocyclops thermocyclopoides* and *Bacillus thuringiensis* subspecies *israelensis*, and permethrin-treated lethal ovitraps. The program was able to reduce the number of *Aedes* mosquitoes and the Immunoglobulin G and Immunoglobulin M seroprevalence in school children (study population) while the control area had an increase in dengue seroprevalence. There were also no reported cases of dengue during the intervention. In the preceding and subsequent years the incidence of dengue was 217.9 and 322.2 cases per 100,000 persons, respectively.

In addition to the aforementioned applications, GIS has been used for cost analysis decisions in vector control planning. GIS software was used to remotely identify probable mosquito habitats around two U.S. military bases in the Republic of Korea using Landsat satellite imagery.[144] This information could then be used to estimate the cost of treating the habitats with larvicides and compare this to the cost of chemoprophylaxis in order to determine which method was more cost effective for controlling malaria. Although we currently lack a chemoprophylactic agent for dengue, this method could still be used to compare various methods of dengue control.

Of particular interest for this study is the use of GIS software to explain and predict the incidence of dengue based on associations with environmental and ecological conditions. While a susceptible individual and an *Aedes* mosquito vector are both required for natural dengue transmission, their presence or absence does not fully explain the variation in dengue incidence between various communities and geographic regions. For example, despite the presence of *Ae. aegypti*, and other *Aedes spp.* vectors throughout the southern portion of the United States, endemic dengue transmission within the country is limited to the border between Texas and Mexico.[145,146] As previously mentioned, other factors such as income, temperature, and rainfall are also associated with dengue transmission.[50-52,147-149] Seasonal fluctuations of dengue in Puerto Rico are related to rainfall and fluctuations in temperature can explain inter-year variability.[150] Although locally obtained weather data is ideal, remote sensing can be also used to predict weather patterns.[151] GIS was used in Barbados to develop a predictive model for dengue using the environmental variables of temperature, rainfall, vapor pressure, and wind speed.[152]In Malaysia, GIS software was used to determine that container density, housing density, and ovitrap results were significantly associated with a dengue outbreak.[153] GIS was also used in Thailand to examine the associations between dengue and rainfall, temperature, humidity, and land usage (water, urban, agriculture, forested) and develop a model for predicting dengue incidence.[154] The coefficient of determination (R^2) for a one month, time-lag model (dengue incidence as a result of climate variables one month earlier) was 0.81 and the study also found that built-up (urban) areas were at higher risk for dengue than the other land usage categories. While it is likely that individual GIS models must be developed for each specific region, the successful development of a model can help dengue program planners identify potential dengue outbreaks before they occur, which may be useful for preventing or mitigating the situation.

Purpose of This Study

The first part of this study examined the effectiveness of a miniaturized Vietrap (funnel trap) in sampling *Ae. aegypti* larvae. Miniaturization of the Vietrap was necessary due to the small opening size of septic tank access ports. The previously validated funnel traps were 18 cm or larger in diameter, but the access ports on the septic tanks in Playa/Playita were as small as 10.2 cm in diameter. It was necessary to test the miniaturized funnel trap in order to assess its ability to detect larval presence and determine if it was suitable for use in the field. I evaluated the miniaturized funnel using a pool with a fixed depth and diameter to assess the effect of larval population density and instar stage on the trap's effectiveness in detecting larval presence. Three replicates were run for both 3rd and 4th instar stages at four different larval population densities in order to develop a model for predicting the total larval population based on trap counts.

The second part of this study was conducted to document *Ae. aegypti* larval presence in septic tanks containing raw sewage. Although the previous CDC study demonstrated the importance of septic tanks as refugia for adult *Ae. aegypti,* questions remained regarding the suitability of septic tank waters for larvae. Current CDC recommendations for controlling larval habitats do not recognize septic tanks as larval habitats and are instead directed towards eliminating standing water in artificial, surface containers.[155] Determining whether the larvae are, in fact, present in septic tanks can help redirect efforts to controlling them or, in the event the larvae are not present in septic tanks, can help ensure the limited financial resources of mosquito control programs are spent efficiently and not misdirected. I hypothesized that the large number of trapped adults were due in part to the presence of larvae in the septic tanks. I further hypothesized that this presence was associated with the tank environmental and water quality factors as previously discussed. Identification of those factors associated with larval presence may help direct subsequent control measures.

The third part of the study attempted to control *Ae. aegypti* mosquitoes within septic tanks. This study, and the previous work by the CDC, demonstrated the significance of septic tanks as refugia for adults and immature *Ae.aegypti*.[82] Since removal of the septic tanks (source reduction) is not an option, I examined whether a 2,2 dichlorvos treated strip (Amvac Chemical Corp; Los Angeles, CA) was a cost effective control for mosquitoes within the septic tanks. Each strip is impregnated with 65 grams of 2,2-dichlorovinyl dimethyl phosphate (dichlorvos) and is intended for use in an enclosed space. Each individual strip costs approximately five USD and is designed to treat a 25 – 34 m^3 (900-1200 ft^3) area for four months. Although not specifically labeled for use in septic tanks, the tested use of the 2,2 dichlorvos is consistent with that specified on the insecticide label.

The final part of the study used geographic information systems (GIS) to analyze associations between dengue incidence and local environmental and socioeconomic variables.[138,154] The ability to remotely predict dengue incidence and identify problem communities is useful for targeting and directing mosquito control programs. I hypothesized that dengue incidence in southeastern Puerto Rico could be remotely predicted at the U.S. Census block group level using elevation (as a surrogate for temperature), income, population density and several other environmental variables. I also tested the hypothesis that *Ae. aegypti* larval presence in septic tanks was associated with dengue incidence by using the absence of sewerage, as established from sewerage maps, to determine which Census block groups primarily used septic tanks, and compared these with georeferenced dengue case reports from the CDC.

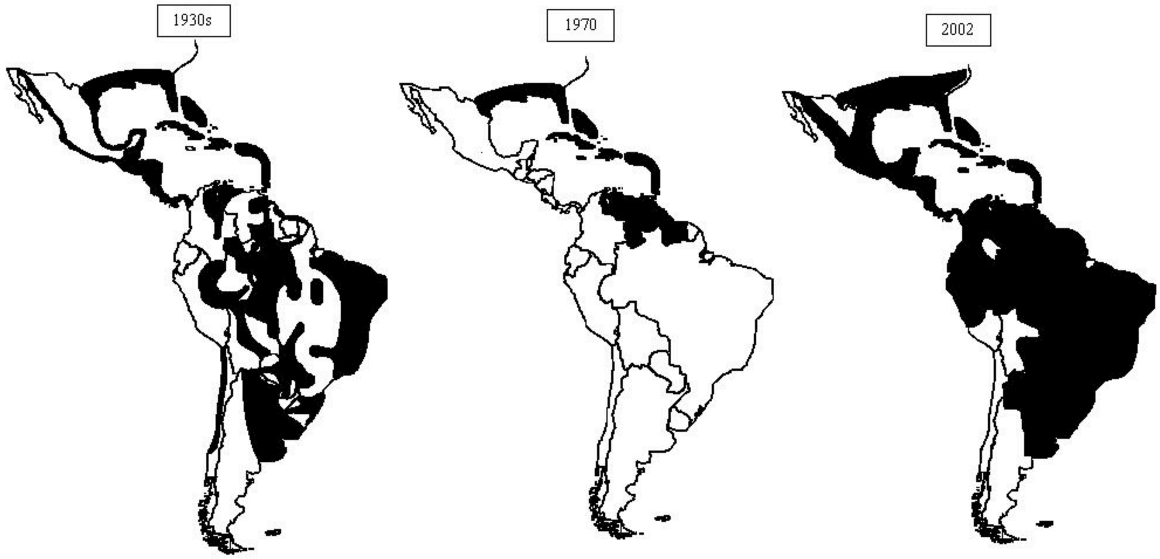

Figure 1. Distribution of *Aedes aegypti* in the Americas.[19]
Reproduced with the permission of the Pan American Health Organization (PAHO). This slide was originally published in the PAHO's product: "Situation Report. Dengue: How are we doing?" To obtain information about PAHO publications please visit their website at: http://publications.paho.org

	Transmission threshold by initial seroprevalence of antibody		
Temperature (ºC)	0%	33%	66%
22	7.13	10.7	23.32
24	2.20	3.47	7.11
26	1.05	1.55	3.41
28	0.42	0.61	1.77
30	0.10	0.15	0.30
32	0.06	0.09	0.16

Table 1. Estimated number of *Aedes aegypti* pupae per person required to result in 10% or greater rise in seroprevalence of antibody to dengue during the course of a year resulting from 12 monthly viral introductions of single viremic individual, the Monthly Introduction threshold.[97]
Used with permission. Original source: Focks DA, Brenner RJ, Hayes J, et al. Transmission thresholds for dengue in terms of *Aedes aegypti* pupae per person with discussion of their utility in source reduction efforts. *Am J Trop Med Hyg* 2000; 62:11-18.

Chapter 2 - Materials and Methods

Validation of Miniaturized Funnel Trap

Modification of methods: The original funnel for the Vietrap was non-standard, purchased through a Vietnamese marketplace, and could not be obtained. In preliminary testing, a close approximation of the Vietrap was constructed using available materials in order to compare it to the miniaturized Vietrap. It was later determined that these modifications, although slight, may have impacted the trap performance, which would prevent comparisons between the miniaturized Vietrap and the original Vietrap. The methodology was then modified to focus solely on the performance of the miniaturized Vietrap. Several changes were made based on the results from the first set of trappings and are addressed below.

Preliminary Testing

Funnel traps: The miniaturized Vietrap was constructed by drilling a hole in the lid of a 120 mL plastic urine specimen cup (Convidien; Mansfield, MA) where the tip of a polypropylene funnel (Thermo Fischer Scientific Inc.; Waltham, MA) was inserted. Each funnel was 11.5 cm high, including a 4.5 cm stem, with a 10 cm mouth and 1 cm stem opening. The funnel was held in place with two size four, 0.95 cm (3/8 in) screws which were partially inserted into the funnel neck. A 3.65 x 0.48 cm steel locking washer was placed around the neck of the funnel as a counterweight, prior to the funnel's insertion through the lid. When filled, the trap had an 11.5 cm draft and an overall height of 14 cm. (Figure 2Figure 2).

The modified Vietrap was constructed using the previously described methods, with several slight modifications [106]. The locking washer described above was used instead of the 20 mm section of galvanized pipe. Also, the funnel in our study was likely a thicker and heavier material than the original funnel as two 0.05 m^2 sheets of transparent plastic bubble wrap, placed around the tip of the funnel, were needed to sustain trap buoyancy and maintain a draft of 18 cm.

The specimen cup was filled to four-fifths capacity with tap water and lowered into the experimental pool water described below. The trap inverted upon entering the water, allowing larvae to swim up through the funnel and subsequently become trapped within the cup. Traps were checked once every 24 hours.

Mosquito larvae: Larvae were hatched from *Ae. aegypti* Rockefeller strain eggs from the CDC insectary in San Juan, Puerto Rico. The larvae were reared in 475 mL plastic cups (50 larvae per cup) for three days and counted prior to transferral to the pool described below. Larvae were predominately 2^{nd} instars, but were not a homogenous population as some 3^{rd} instars were present. The larvae were kept in the pool for 48 hours before they were removed and a new batch was transferred into the pool.

Pools: In the preliminary tests, a 1.5 m diameter plastic wading pool was used for larval trapping. The pool was filled with tap water and left at room temperature for one hour prior to introducing the larvae. The depth of the water was 24 cm. The laboratory thermostat was set at 23.88 ºC (75 ºF) in an attempt to maintain the water at an approximately constant temperature. The water temperature was 22.38 ± 0.65 ˚C. The pool was covered with white canvas fabric to minimize disturbance of the traps and larvae. Larvae were removed every 2 days and the water was changed half way through the experiment. Both the miniaturized Vietrap and the modified Vietrap were placed in the same pool. Five trap replicates were run for each larval population density of 0.011 (200 larvae), 0.022 (400 larvae), 0.033 (600 larvae), and 0.055 (1000 larvae) larvae / cm^2.

Final Testing

Mosquito larvae: When the methodology was modified three months later, the larvae were reared as above, but were sorted according to instar stage prior to placement in the pools. During the addition of these larvae, the pool was visually divided into four quadrants and equal numbers of larvae were added to each quadrant in an attempt to minimize clustering of the larvae in just one area. Third and fourth instar larvae were kept in the pools for 24 hours before they were removed and a new batch was transferred into the pool. Third and fourth instars were introduced to separate pools.

Pools: In the final tests, the depth of the water was 24 cm. As before, the laboratory thermostat was set at 23.88 ºC (75 ºF) in an attempt to maintain the water at an approximately constant temperature. The temperature was 20.43 ±0.52 ˚C which was slightly lower than in the preliminary testing, most likely due to the difference in seasons and the switch between air-conditioning and heating of the room. Two identical pools were used side by side, and the larvae were rotated between each pool (i.e. Day 1 – 3^{rd} instars Pool A, 4^{th} instars Pool B; Day 2 – 3^{rd} instars Pool B, 4^{th} instars Pool A). The pools were left uncovered so as not to restrict air currents and subsequent trap movement within the pool, and to allow for the casting of shadows as people passed through the lab. The effect of shadow-casting on larval diving behavior was done to mimic the effect of water disruption from toilet flushing on diving behavior which would presumably occur in the field. The pools were placed directly under overhead fluorescent lighting which was turned off each evening. Each pool had one miniaturized Vietrap. Three trap replicates were run for each larval instar stage at each population density of 0.011, 0.022, 0.033, and 0.055 larvae per cm^2.

Data analysis: Stata/IC 10 (StataCorp LP; College Station, TX) was used for statistical analyses. Daily trap counts were converted to a percentage of the total larval population. The Wilcoxon rank sum test was used to compare the trapping percentages between larval instar stages. A non-parametric trend test was used to assess the effect of population density on trap performance. Simple linear regression lines were used to extrapolate expected trap catches based on larval population densities. Trap catch percentages were calculated for each density and instar stage and a Kruskal-Wallis equality-of-populations rank test was used to examine the differences between trap percentages in order to assess the interaction between larval stage and population density.

Field Evaluation of *Aedes aegypti* Larval Presence and Abundance in Septic Tanks of Puerto Rico

Study objectives: The primary objectives of this portion of the study were to document the presence of *Ae. aegypti* larvae in septic tanks, and determine what water quality factors or septic tank environmental variables were associated with larval presence and abundance. To accomplish the first objective, a miniaturized Vietrap was used to sample septic tank waters in Playa/Playita, Puerto Rico for the presence and abundance of *Ae. aegypti* larvae. For the second primary objective, I used a survey of water usage and septic tank maintenance, physical and observational measurements of the septic tank and surrounding environment, and *in situ* water sampling.

Adult emergence trapping was a secondary objective of the study. While the miniaturized Vietrap was an effective tool for sampling larval populations under laboratory conditions, its effectiveness in the field (e.g. septic tanks) was unknown. Of particular concern, was the potential for floating sewage to obstruct the funnel mouth, thereby blocking larvae from entering the reservoir and becoming trapped. The presence of adult mosquitoes was therefore selected as a potential proxy for larval presence. Baseline data on adult presence and abundance in each septic tank was collected and then the tanks were treated with an insecticide to kill any resting adults within the septic tank. Two additional emergence trappings were then performed on each septic tank. As the septic tanks were sealed it was assumed that few new adults could enter the septic tank. Additionally, since it was also assumed that the insecticide treatment would kill most of the resting adults in the septic tank, the majority of the captured adults in the emergence traps were likely a result of larval/pupal development within the septic tank.

<u>Selection of septic tanks</u>: The community of Playa/Playita (1400 households) is located on the southern coast of Puerto Rico (17º58’N, 66º 18’W) within the municipality of Salinas.[82] (Figure 4). The mean annual rainfall is 973 mm and is seasonal, with a dry season between December and April.[84,156] Mean annual temperature is 26.6 ºC. There are approximately 1,350 structured premises, 84% of which are characterized as households.

The digitized map of streets and buildings within the community, which was used in the previous study, was obtained from the CDC as a means of numerically identifying individual homes, and septic tanks. The average household size is between 3.04 – 3.15 persons which equates to nearly 3,500 total persons living in the community.[157] Between September 1998 and January 2008, there was a total of 116 laboratory confirmed cases of dengue fever within the community (CDC, unpublished data). Forty-six (40%) of the confirmed cases were from the 2007 dengue epidemic.

Septic tanks in Playa/Playita do not have leach fields and consist solely of a holding tank that is professionally serviced and pumped on an as needed basis. A sewerage system was installed over two years ago; however, there are still homes that use septic tanks. Sampling was conducted during the dry season (mid February – early April) in order to minimize the potential for rainwater dilution within the septic tanks.

Surveys were attempted at all eligible homes within the community. Two neighborhoods exclusively used the sewerage system and were therefore excluded. Public buildings, businesses, and multi-family structures were also excluded. Residences were excluded if they were connected to the sewerage system, the septic tank could not be accessed, the septic tank lacked standing water, or the owner did not wish to participate. Written, informed consent was obtained at each household from a person over 18 years of age prior to each survey.

<u>Funnel trapping for larval surveillance</u>: Miniaturized Vietraps were constructed using the method described above. The specimen cups were filled to fourth-fifths capacity with tap water and lowered into the septic tank using two, 3 meter fishing lines

(9 kg strength) that were tied to the funnel's mouth. The traps inverted upon entering the water which allowed larvae to swim up through the funnel and subsequently become trapped within the cup. Funnel traps were placed during the morning and retrieved the following day for four consecutive days.

Water usage and septic tank maintenance: Closed-ended surveys were conducted on water usage and septic tank maintenance at each enrolled home. Survey questions were asked of the same individual that provided consent, generally the homeowner. Questions regarding water usage included the number of occupants in the home, shower and bath usage (e.g. frequency, duration), washing machine usage, and water source (Appendix 1 – Water and Septic Tank Usage). Questions pertaining to septic tank maintenance included the source of the septic water, frequency of service, size, construction material, and age of the tank. All questionnaires and research materials received approval from the Uniformed Services University (USU) Institutional Review Board office prior to their use.

Septic tank water properties and environment: The surface area, distance to home, vent pipe length, and opening distance from the wall of the tanks, and above ground height were measured for each tank (Appendix 2 – Field Observations). Screening of the vent pipe, cracking of the walls, sun exposure, and opening coverage were visually noted. Water properties were measured at the water's surface using a pH/CON 300 meter (Oakton Instruments; Vernon Hills, IL) to determine pH, temperature, TDS, and conductivity for four consecutive days. Probes were calibrated daily. Samples were measured *in situ* due to safety concerns.

Insecticide treatment: An insecticidal treatment was used to eliminate adult mosquito populations after adult emergence trapping for one day. This was done after sealing the tanks with a foam sealant, and was performed in an effort to assess adult emergence rates from the tank habitat. A 2,2 dichlorvos impregnated strip was placed in each tank on Day 2 for three to four hours to kill the resting adults which were present after the tank was sealed so that emergence traps were likely to contain only newly emerged adults. Strips were laboratory tested in three, 125 L (33 gal), new plastic garbage containers prior to use in the field. Twenty-eight adults were placed in emergence containers inside the closed 125 L containers along with approximately 20 L of tap-water. Twenty-one larvae and three pupae were also added to the water. After one hour of exposure, 93% of the adults and 10% of the larvae were dead. Three hours of exposure resulted in 100% adult and 50% larval mortality. All of the pupae were alive and mobile after 24 hours of exposure to the insecticide. Due to a lack of time, the laboratory evaluation of the strip lethality was not repeated prior to field use; however, the strip lethality was monitored in the field by using 2,2 dichlorvos impregnated strips to kill adult emergence trap samples.

Emergence trapping: Sealing of the septic tanks and emergence trapping was conducted according to the previously described method using a screened one-gallon container placed over the tank openings.[82] Baseline emergence traps were set on Day 1 and collected the next morning. An emergence trap was placed over each septic tank on Day 2, after removal of the 2,2 dichlorvos impregnated strip to confirm the insecticidal activity by checking to see if the number of captured adults was reduced from baseline values. Tanks were sealed on Day 3 when the traps were not used. A final emergence trapping was conducted on Day 4 to see if the number of captured adults increased after removal of the insecticide.

Processing of samples: Adults were killed by placing the emergence traps and a 2,2 dichlorvos impregnated strip in 0.68 m^3 space for three hours. Mosquitoes were identified using the standard dichotomous keys.[158] Larval samples were killed in boiling water and preserved in 95% ethanol. Voucher specimens were sent to the Walter Reed Biosystematics Unit. Live pupae from the traps were manually transferred to emergence vials with a pipette. The pupae were reared in a shaded, outside location under ambient air conditions and emerged adults were identified as above.

Data Analyses: Stata/IC 10 was used for statistical analysis. The dependent variables of interest were larval presence, larval abundance, adult presence, and adult abundance. The independent water usage and quality variables were the number of occupants, shower length and frequency, washing machine usage and frequency, source of potable water, source of septic tank water (e.g. toilet only, toilet and bath), pH, temperature, TDS, and conductivity. The independent septic tank environmental variables were frequency of septic tank service, surface area, distance between septic tank and house, distance between tank access port and tank wall, above ground height, presence of cracks in tank wall, sun exposure, and coverage of the access port. The exposure variable (time) for negative binomial regression was the number of days an emergence trap or miniaturized Vietrap was used in each septic tank.

Negative binomial regression was used to examine the relationship between adult and larval mosquito counts and the individual independent variables due to over-dispersion of the data. Those variables which were found to have an individually (unadjusted) significant association with trap counts were included in a full model using backward-stepwise regression (entry 0.05, removal 0.1) to develop a final model for predicting mosquito counts.

Adult and larval presences were examined as single entities, and were not divided into pre and post insecticide treatment. That is to say, there was no distinguishing between presence on Day 1 versus presence on Day 4, rather a positive presence was defined as larval or adult presence on one or more days. Logistic regression was used to examine the relationship between adult or larval mosquito presence and the individual independent variables. Individually significant variables were then examined using multivariate, backward-stepwise, logistic regression (entry 0.05, removal 0.1) in order to develop a model for predicting mosquito presence. A Fisher's exact test was used to examine the association between adult presence and larval presence.

Control of *Aedes aegypti* Mosquitoes in Septic Tanks

Sample selection: The sampled septic tanks in Playa/Playita were all sealed and screened as part of the investigation into larval presence in septic tanks. Since this action limited mosquito entry into the tanks, they were not ideal samples for testing the effectiveness of the 2,2 dichlorvos insecticide in controlling mosquitoes within septic tanks. The community of Las Mareas, Puerto Rico was selected based on its proximity to

Playa/Playita (< 5 miles) and use of septic tanks. Informed consent was obtained from homeowners prior to enrollment in the study. Streets were randomly selected and all homes on the street were solicited for enrollment into the study until a total of 30 accessible septic tanks were identified.

Emergence trapping and insecticide treatment: An emergence trap was placed overnight on each of the 30 septic tanks using the methods described above. No sealing of cracks or screening of vent pipes was performed. Adult *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes were collected from 25 septic tanks. The 25 tanks that were positive for mosquito presence were randomized and a 2,2 dichlorvos impregnated strip was placed in 12 of the septic tanks. The remaining 13 septic tanks served as controls. Impregnated strips were left in place for four months. Overnight emergence trapping was conducted on each septic tank once a month for four months.

Processing of samples: Adults were killed by placing them in a freezer (- 10 ºC) for 30 minutes. Mosquitoes were identified using the standard dichotomous keys.[158]

Data Analysis: Stata/IC 10 was used for statistical analysis. A Wilcoxon rank-sum test was used to assess the monthly change in trap counts from septic tanks between the two groups.

Use of GIS to Examine Associations Between the Incidence of Dengue and Environmental and Socioeconomic Conditions

I conducted a retrospective ecological and socioeconomic analysis of suspect and laboratory confirmed cases of dengue virus in seven Puerto Rican municipalities between March 2003 and April 2008. This data was obtained from the CDC's dengue case database which is maintained by Dengue Branch in San Juan, Puerto Rico. Patient names, ages, and sex were removed prior to release of the data. Serum samples of suspected cases were submitted by local attending physicians to the Dengue Branch laboratory in San Juan, PR. Laboratory confirmed cases were identified as dengue virus (acute specimen with positive PCR or tissue culture) or as unidentified, but most likely dengue, Flavivirus (IgG titer ELISA antibody > 160 in acute serum, ELISA IgG antibody > 163,840 in convalescent serum, MAC-ELISA IgM positive in acute specimen, or seroconversion in paired specimens). Georeferencing of cases was performed by SeekData, Inc. (Kissimmee, FL) using patient home addresses, as reported by submitting physicians.

<u>Sample selection:</u> The municipalities of Cayey, Coamo, Guayama, Humacao, Juana Diaz, Ponce, and Santa Isabel were selected based on their geographic location and population size. (Figure 5Figure 4). The municipalities were located in the southeast portion of Puerto Rico where there is a noticeable dry season (December – April). The population of the municipality capital (≥ 4,500) was used to ensure there were multiple urban block groups with and without sewerage lines in each city for comparison.

<u>Selection of environmental and socioeconomic variables:</u> Population demographics were obtained from the U.S. Census Bureau using American FactFinder.[157] The total population, urban percentage, number of households, income, and average household size

for each block group were downloaded from the Census 2000 Summary File 1 and Summary File 3. Block groups are the smallest unit of interest for which all variables were available. Topologically Integrated Geographic Encoding and Referencing (TIGER) files of block group maps were also downloaded from the Census website. The locations of area hospitals were obtained from the ArcGIS 9 ESRI Data and Maps (ESRI; Redlands, CA) series as I theorized that underreporting may have occurred, especially in remote areas. Altitude has been shown to be an important predictor of air temperature in Puerto Rico with higher elevations having cooler temperatures.[151] Shuttle Radar Topography Mission (SRTM) elevation data (90 m resolution) was obtained from the CGIAR Consortium for Spatial Information website.[159] ArcGIS 9.2 (ESRI; Redlands, CA) was used to calculate the area, mean distance to the nearest hospital, and mean elevation for each block group. Precipitation data was not available at the block group level so the reported monthly average of the municipality capital was used for each municipality.[160] The association between rainfall and dengue geographic incidence was thus not examined at the block group level; however, I did examine whether the associations between dengue incidence and the other variables of interest varied by season (e.g. wet or dry). The monthly rainfall for the region was the un-weighted mean of the seven municipalities.

<u>Sewerage lines:</u> Absence of sewerage lines was used to identify locations using septic tanks. I assumed that populated areas which did not have a municipal sewerage system utilized septic tanks for their waste. As the amount of sewerage coverage within a block group decreased, I assumed the frequency of septic tank usage in homes increased.

Maps of sewerage systems were obtained from the Puerto Rico Aqueduct and Sewer Authority (San Juan, PR) in a portable document format (PDF) and converted to a tagged image file format (TIFF) using Bluebeam PDF Revu Standard Edition (Pasadena, CA). PCI Geomatica (Ontario, Canada) was used to count the number of sewerage pixels per block group.

Data Analysis: Stata/IC 10 was used for all statistical calculations. Negative binomial regression was used due to over dispersion of the data. The dependent variables of interest were dengue incidences, by Census block group, during the rainy season (May – November), the dry season (December – April), and the period of March 2003 – April 2008 as a whole. The independent variables of interest were the mean household size, mean distance to the nearest hospital, mean elevation, mean number of households, mean income, the number of sewerage pixels, and the urban percentage of each Census block group. Unadjusted and adjusted incidence rate ratios were calculated for each independent variable. A backwards, stepwise, negative binomial regression (entry = 0.5, exit = 0.1) was used to examine the association between the number of cases of dengue per block group and the independent variables of interest in order to develop a model for predicting the incidence of dengue within the region. The covariance between independent variables was examined using Pearson’s correlation.

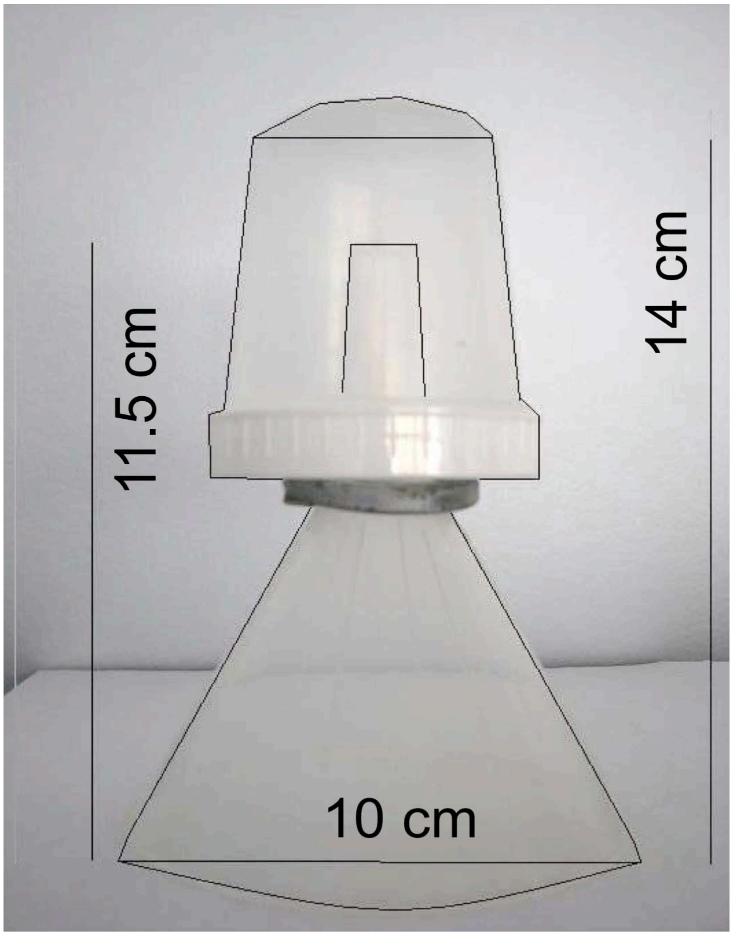

Figure 2. Miniaturized Vietrap funnel.

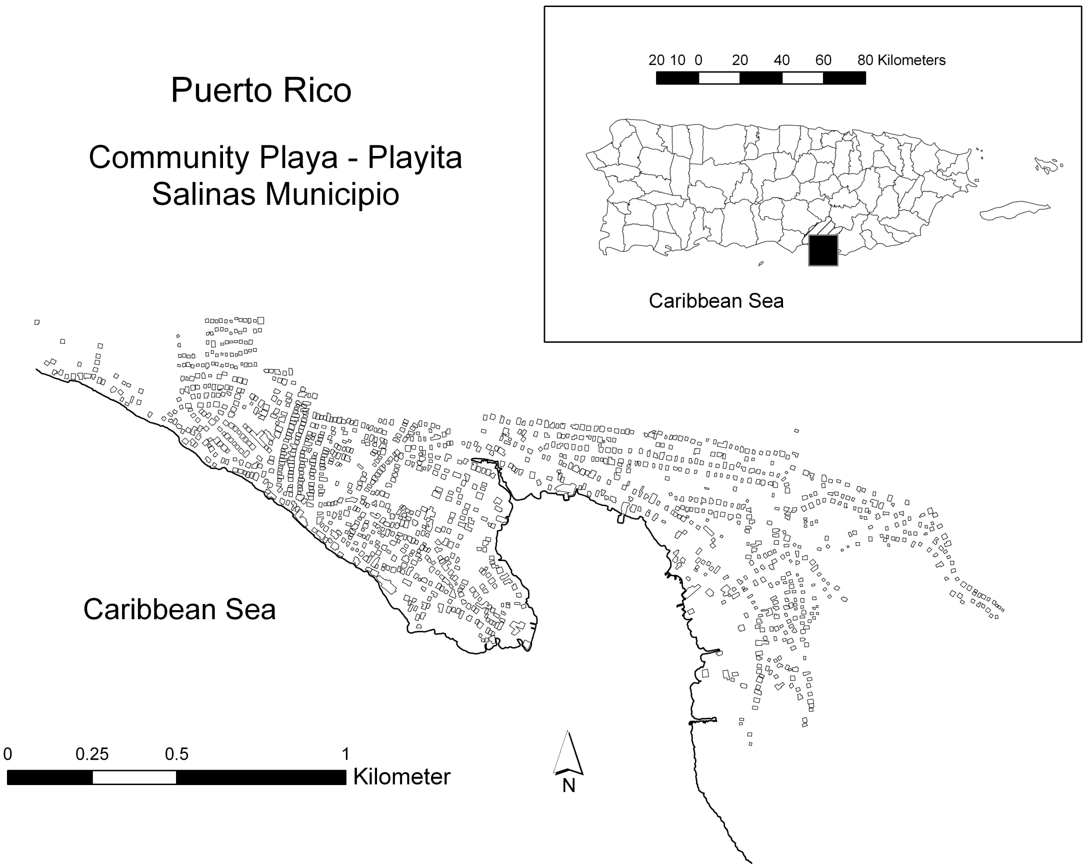

Figure 4. Geographic location and layout of Playa/Playita, southeast Puerto Rico.[84] Used with permission. Original source: Barrera R, Amador M, Clark G. Use of the pupal survey technique for measuring *Aedes aegypti* (Diptera: Culicidae) productivity in Puerto Rico. *Am J Trop Med Hyg* 2006;74:290-302.

Figure 5. Geographic location for the municipalities of Cayey, Coamo, Guayama, Humacao, Ponce, and Santa Isabel, southeast Puerto Rico.

Chapter 3 - Validation of Miniaturized Funnel Trap

Results

In preliminary laboratory studies, the miniaturized Vietrap detected larval presence (at least one larvae in the trap reservoir) 80% of the time with a population density of 200 larvae (0.011 larvae per cm^2) and the modified Vietrap detected larval presence 60% of the time at the same population density. Both traps detected larval presence 100% of the time at population densities of at least 0.022 larvae per cm^2 (400 or more larvae). The mean trap percentage (larvae trapped from a known population) for the miniaturized Vietrap (4.9%) was greater than the mean trap percentage for the modified Vietrap (3.8%); however, this difference was not statistically significant at generally accepted probability levels ($p > 0.0534$). (Table 2). There was a significant trend (nonparametric trend test, $z = 2.45$, $p < .015$) of increasing percentage trapped with higher larval population densities with the miniaturized Vietrap, but not with the modified Vietrap (nonparametric trend test, $z = 1.25$, $p > .21$). Despite the differences in trends, there was no significant difference in the trapped percentage of the larval population between the two traps at any of the four larval population densities (Kruskal-Wallis, chi-squared probability ≤ 5.101 with 3 degrees of freedom, $p \geq 0.16$).

In the final, modified study, the miniaturized trap detected larval presence 83% of the time at a population density of 0.011 larvae per cm^2 (200 larvae in the pool) and detected larval presence 100% of the time at population densities of at least 0.022 larvae per cm^2 (400 or more larvae in the pool). (Table 3). There was no significant difference in the number of larvae trapped between 3rd and 4th instars (Wilcoxon rank-sum, $z = 0.26$, $p > 0.795$). There was a significant (nonparametric trend test, $z \geq 2.08$, $p \leq 0.037$) trend of increasing percentage trapped with higher larval population densities for both larval stages. The interaction between larval population and larval stage was not significant (Kruskal-Wallis, chi-squared probability ≤ 5.821 with 3 degrees of freedom, $p \geq 0.12$). In other words, the effect of larval population density on trap percentage was not affected by the larval instar stage.

Discussion

These results indicate that the miniaturized Vietrap is an effective surveillance tool for sampling larval populations in the laboratory environment, despite having a smaller funnel opening than the original trap. The fact that the trap has a shallower draft may offset the smaller opening as larvae do not have to dive as deeply to get under the trap.[106] Although not significant, the slightly decreased trap effectiveness with 4th instars, as compared to 3rd instars, may be a result of recent pupation. Although only 4th instars were introduced into the pool, a small number of pupae were present in the 4th instar pool 24 hours later, when the funnel trap was collected. The diving activity of pupae is less than the diving activity of larvae since the pupae are not foraging for food. The limited diving of pupae reduces the likelihood of them diving below the funnel mouth and becoming trapped.

I hypothesized that the trap sensitivity (ability to detect larval presence) would improve with higher larval populations, but the increasing trapped percentage (of the total population) with higher larval densities was unexpected. This may be due to the clustering behavior of the larvae. Under laboratory conditions *Ae. aegypti* larvae were noted to congregate in small sections of the rearing pans.[161] With low numbers of larvae, they may cluster together in one or two small areas and the floating funnel trap may not remain adjacent to the larvae long. With higher numbers, the larvae may cluster over a larger area or in more locations which could increase the time the trap remains in an area of the pool containing larvae.

The data from the miniaturized Vietrap can be used to develop regression plots for larval trap counts as a function of the total population. Since the trap differences between 3rd and 4th instars were not statistically significant, the results were combined to form a single regression line, $R^2 = 0.62$. (Figure 7). This regression line can then be used to estimate the total larval population, under similar conditions, of a sampled container using the trap results.

Limitations of this study: Due to safety and aesthetic concerns, the trap was tested in clean water instead of sewage, but several environmental factors of septic tanks could impact the calculations. Without testing the floating trap in conditions similar to those found in a septic tank, it is difficult to determine how the trap would perform. Water conditions in septic tanks could increase or decrease trap performance. For instance, water entering the tank (e.g. after flushing a toilet) will disturb the surface and cause the larvae to dive. Frequent disturbance of the water, as seen in high occupancy homes, could increase larval vertical movement and trap performance. On the other hand, the presence of floating sewage which could decrease the trap effectiveness through obstruction of the funnel opening or limiting the trap's horizontal movement to a small section of the tank. This study cannot determine whether these two conditions equally offset each other, or whether they, or another unidentified factor, shift the true trap performance away from the results seen in control conditions under laboratory settings. As such, regression values should be viewed as an approximation of the total larval population in septic tanks rather than an exact number.

Another limitation of the study was the inability to construct a Vietrap according to the original specifications. As a result, it was not possible to make direct comparisons between the miniaturized Vietrap and the original Vietrap as described by Russell and Kay.[106] Nonetheless, some general observations can still be made. The fact that the narrower miniaturized Vietrap performed as well as the wider, modified Vietrap is likely a result of the shallower draft. Although the miniaturized Vietrap covered a smaller area, a mosquito larva need only dive 11.5cm to enter the funnel opening and become trapped versus the 15.5cm needed to enter the modified Vietrap. Similar observations were made when comparing the original Vietrap against the broader and deeper Austrap.[106]

In the first study, the percentage of larvae trapped from a known population was significantly higher ($p = 0.022$) during the second 24-hour period than during the first 24-hour period. The increased performance may be a result of the increased vertical movement at the older stages.[106,162] For *Anopheline* larvae, diving depths due to changes in water quality were greater for 4th instars than 2nd instars and similar behavior may occur in *Ae. aegypti* larvae as well.[163] An increase in vertical movement would increase the likelihood of the larval instars entering the funnel and becoming trapped. Unfortunately, the larvae were not staged between the two time periods so it cannot be said for sure whether this difference was a result of instar stage or another factor.

Another limitation of this study is that it did not determine the minimum larval population density threshold required for trap detection. In the final testing of the miniaturized Vietrap, priority was given to larger population densities in order to develop a more accurate regression plot for determining larval population densities from trap counts. Unfortunately, with larval population densities that are 0.11 larvae / cm^2 or less, there is a chance ($\geq$ 17%) that the miniaturized Vietrap would fail to detect their presence. This chance can be reduced, but not eliminated, by repeat trapping.

A final limitation was the difference in methodology between the two studies. While this prevented the direct comparison of trap results from 2nd instars with 3rd or 4th instars, it does not affect the conclusions drawn from the final study. Additionally, larval taxonomy keys are generally for 4th instars so regression lines for the mature stages are likely of more benefit than 1st or 2nd instar regression lines.

Larval Population	**Larval Density (per cm^2)**	**Miniaturized Vietrap (Percent)**	**Modified Vietrap (Percent)**
200	0.011	3.8 ± 2.8 (2)	9.0 ± 11.2 (5)
400	0.022	21.3 ± 14.3 (5)	8.1 ± 7.6 (2)
600	0.033	23.9 ± 21.3 (4)	25.6 ± 29.0 (4)
1000	0.055	86.2 ± 43.1 (9)	53.6 ± 66.4 (5)

Table 2. Mean number of *Aedes aegypti* larvae trapped (± S.D.) and percentages of total larval population trapped by a miniaturized Vietrap and modified Vietrap at varying larval densities in a 1.52 m (diameter), indoor, and covered pool.

Larval Population	Larval Density (per cm^2)	3rd Instars (Percent)	4th Instars (Percent)
200	0.011	11.7 ± 8.7 (6%)	2.7 ± 1.5 (1%)
400	0.022	40.3 ± 28.0 (10%)	39.0 ± 26.1 (10%)
600	0.033	84.0 ± 32.0 (14%)	71.7 ± 12.1 (12%)
1000	0.055	260.3 ± 88.4 (26%)	162.0 ± 39.9 (16%)

Table 3. Mean number of 3rd and 4th instar *Aedes aegypti* larvae trapped (± S.D.) and percentages of total larval population trapped by miniaturized Vietrap at varying larval densities in a 1.52 m (diameter), indoor, and uncovered pool.

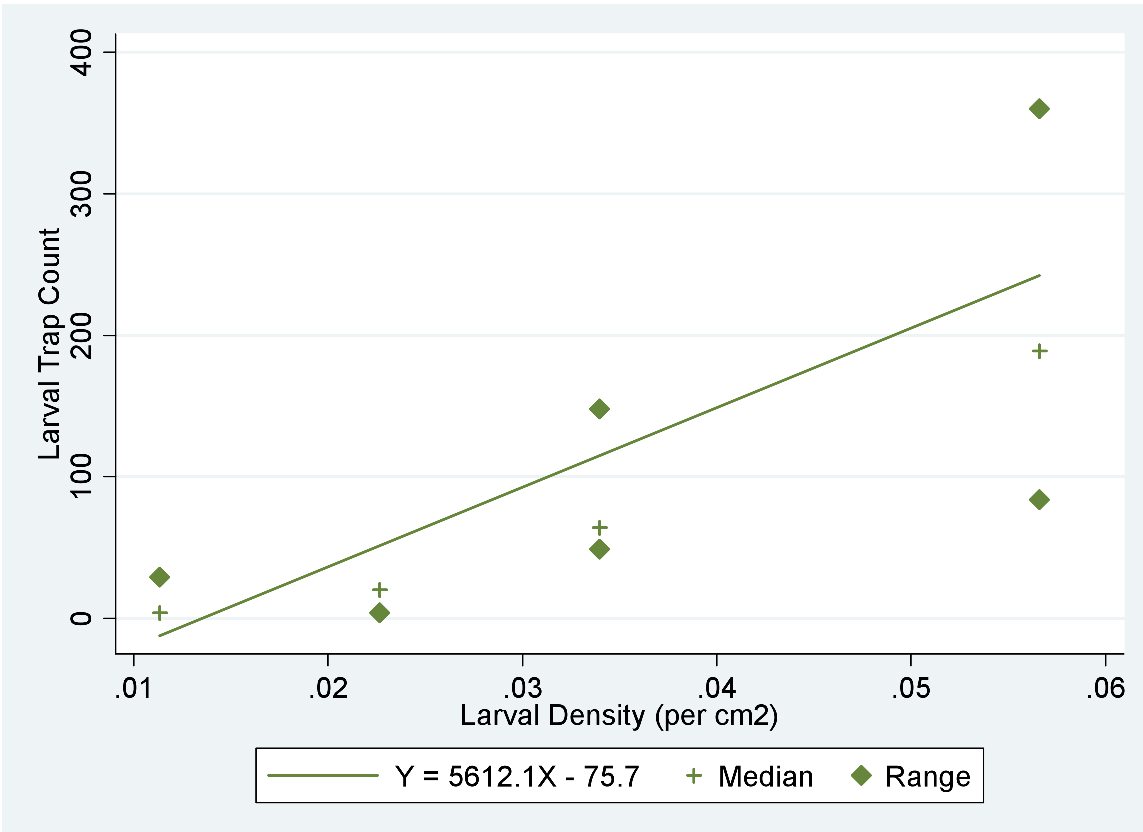

Figure 7. Linear regression plot (R^2 = 0.62) of the number of *Aedes aegypti* larvae trapped in a miniaturized Vietrap, as a function of initial larval density of the sampled population from a 1.52 m (diameter), indoor, and uncovered pool.

Chapter 4 - Presence of *Aedes aegypti* Larvae in Septic Tanks

Results

A total of 1,013 residences were identified for consideration of inclusion into the study. Investigators were unable to interview an adult at 55% of the homes. Of the remaining 460 homes, 23% were connected to the sewer, 39% had a septic tank that was located under the home, permanently sealed, or was otherwise inaccessible, 11% declined to participate, and 5% were dry despite being actively used. Four of the original 100 septic tanks in this study were later found to be dry, leaving the study with a total of 96 eligible residences for examination.

Water usage and tank maintenance: Responses for water usage and septic tank maintenance are summarized in Table 4. One household declined to give an answer for shower use, frequency, and length. No home had a dishwasher.

Septic tank water properties and environment: Results from the septic tank water properties and environment analysis are summarized in Table 5. Nine septic tanks were partially located under the home. Three septic tanks were buried so that it was not possible to measure the outside walls or the distance from walls to the center of the tank opening. The association between screening and mosquitoes was not examined as only 7 of 60 vent pipes were unscreened. Dilution of the septic tank waters was minimal due to the limited rainfall.

Mosquito productivity: Eighty-nine septic tanks were sampled for larval presence as seven septic tanks had an opening that was too small for the funnel trap. (Table 6). *Aedes aegypti* larvae were captured in sixteen (18%) of the septic tanks (mean: 10.28, 95%

confidence interval: -2.34 – 22.90). Three septic tanks could not be adequately sealed, and did not have an adult emergence trap. *Aedes aegypti* adults were found in forty-six (49%) of the septic tanks (mean: 8.67, 95% CI: 3.66 – 13.67). A total of 28 *Ae. aegypti* pupae were recovered from the floating funnel traps of six septic tanks, and were successfully reared to adulthood for identification. Additionally, one adult *Ae. aegypti* mosquito was also recovered from inside a floating funnel trap. Although they were not the species of interest in this study, *Cx. quinquefasciatus* larvae were collected from sixty-six (74.16%) of the septic tanks (mean: 129.62, 95% CI: 64.90 – 194.34) and *Cx. quinquefasciatus* adults were found in ninety (96.77%) of the septic tanks (mean: 155.48, 95% CI: 92.15 – 218.80).

<u>Insecticide treatment</u>: The mean number of captured *Ae. aegypti* adults on Day 1 (5.27 ± 21.38) was significantly reduced on Day 2 (2.55 ± 9.46) after treatment with 2,2, dichlorvos impregnated strips (Paired t test, $t = 1.69$, $p < 0.0471$, degrees of freedom = 90). The mean number of total mosquitoes (*Cx. quinquefasciatus* and *Ae. aegypti*) on Day 1 (237 ± 580.65) was also significantly reduced on Day 2 (76.21 ± 209.79) following septic tank treatment with the impregnated strips (Paired t test, $t = 3.39$, $p < 0.0006$, degrees of freedom = 90). There was no significant difference between the mean number of *Ae. aegypti* or total mosquito counts on Day 1 versus Day 4 (Paired t test, $-0.08 < t < 1.46$, $p > 0.0743$, degrees of freedom = 88).

<u>*Aedes aegypti* presence</u>: At the univariate level there was a positive association between larval presence and cracking of the tank wall or cover and opening status which was defined as open, covered, or capped. (Table 7). Cracking and opening status were

both significant in the final model, although only a small fraction of the variance was explained by the model ($R^2 = 0.11$). Adult presence was positively associated with cracking, uncapped openings, and pH, and inversely associated with the number of occupants. In the multivariate model only the number of occupants (inversely) and pH (positively) contributed to the fit of the model ($R^2 = 0.10$). The presence of adult *Ae. aegypti* mosquitoes in septic tanks was significantly associated with the presence of the larval form (Fisher exact test = 0.004).

Aedes aegypti counts: Larval counts were positively associated with surface area, uncapped openings, and tank age and negatively with above ground height, TDS, conductivity, and the number of occupants. (Table 8). In the multivariate model, larval counts were positively associated with surface area and uncapped openings, and negatively associated with TDS and the number of occupants (Pseudo $R^2 = 0.11$). No *Ae. aegypti* larvae were recovered from septic tank waters with TDS values greater than 1.825 ppt. The larvae were also not recovered from waters with conductivities greater than 3680 μS. This suggests there may be a possible contribution threshold for this factor, but further research is needed to confirm this hypothesis. Larval *Ae. aegypti* counts were also highly correlated with *Cx. quinquefasciatus* larval counts (r = 0.56, degrees of freedom = 87, $p < 0.0001$). The adult counts were positively associated with cracking and negatively associated with TDS, conductivity, the number of occupants, washing-machine use, and septic source. The number of occupants and septic source were significant but only a small fraction of the variance in larval counts was explained by the final model (Pseudo $R^2 = 0.03$). Adult *Ae. aegypti* counts were also significantly correlated with *Cx. quinquefasciatus* adult counts (r = 0.23, degrees of freedom = 91, p = 0.027)

Culex quinquefasciatus presence: *Culex quinquefasciatus* larval presence was inversely associated with TDS and conductivity and positively associated with public water sources. Water source was excluded due to co-linearity with both TDS and conductivity and neither of the two remaining variables contributed to the development of a full model. Logistic regression of adult presence was not performed since the adults were absent in only three tanks.

Culex quinquefasciatus counts: Larval counts were positively associated with cracking of the septic tank walls and negatively associated with TDS individually. Neither variable contributed to the development of a full model. The adult counts were negatively associated with the opening status and shower length. Neither of the two variables contributed to the development of a full model.

Correlation between Independent Variables: Pearson's coefficient was used to examine the correlation between independent variables. Although TDS and conductivity were highly correlated (r = 0.84, degrees of freedom = 94, p = 0.000) both terms were left in the multivariate model as they had opposing effects, and thus did not over-fit the model. There was little correlation (r < 0.25, d.f. = 91, p > 0.05) between the other independent variables in the final models.

Discussion

This study provides strong evidence of *Ae. aegypti* larval presence in septic tanks and marks the first published record of *Ae. aegypti* larvae being recovered from holding tanks containing raw sewage in the Caribbean. Although there was significant variation in trap catches, large numbers of larvae and adults were trapped from some septic tanks on a daily basis. Identifying those septic tanks which are productive, and the factors related to that production, may help with mosquito control strategies.

The significant association between the presence of *Ae. aegypti* larvae and *Ae. aegypti* adults in septic tanks suggests complete development of the mosquito is occurring within septic tank waters. If the mosquito was not developing in the septic tanks we would have expected an approximately equal distribution of the adults between the septic tanks that were positive for larvae and those that were not (Table 9). Instead, *Ae. aegypti* adults were present in 13 of the 15 septic tanks containing *Ae. aegypti* larvae. (An adult emergence trap was not placed on one of the septic tanks containing *Ae. aegypti* larvae; hence, the total of 15 positive septic tanks reported here instead of the 16 septic tanks which were reported earlier).

The hypothesis concerning *Ae. aegypti* development within septic tanks is further supported by the presence of the *Ae. aegypti* adult and pupae within the reservoir of the floating funnel trap. While it is possible that the adult mosquito may have entered the trap during placement or removal of the miniaturized Vietrap, it is more likely that it emerged from its pupal stage while within the trap reservoir. This is supported by the presence of other *Ae. aegypti* pupae within the trap reservoirs, which suggests that both the larval and pupal stages of the mosquito are present in the waters of at least some septic tanks in

Puerto Rico. Pupal mortality rates for *Ae. aegypti* are generally much lower than those of the larvae.[71] If a larval mosquito was able to survive and develop into a pupa, it is probable that the mosquito could complete the process and develop into an adult.

The low number of recovered pupae (28), relative to the number of recovered larvae (661), is likely a result of the limited diving behavior of pupae. During the pupal stage, mosquitoes do not forage for food and diving is often done in response to a perceived threat, i.e. disruption of the surface water due to flushing of a toilet. Floating funnel traps, like the miniaturized Vietrap used in this study, rely on vertical movement of the mosquito. Since the pupae are not diving much, the likelihood of them diving below the funnel mouth opening and subsequently becoming trapped is reduced.

The positive relationship between the mosquitoes and cracking of the tank walls or uncapped access ports is consistent with the results from the previous CDC study that measured adult emergence.[82] Over half of the surveyed tanks had cracks or uncapped tank access ports through which a mosquito could gain entry. Completely sealing these tanks could reduce mosquito access and serve as an effective intervention strategy.

The TDS range in this study, 0.6 – 3.985 parts per thousand (ppt), was significantly higher than the ranges reported in previous studies (0 – 188 parts per million, 0 – 0.188 ppt from natural aquatic habitats), and may explain why there was a negative association with trap counts instead of the previously reported positive association.[75,164] Although TDS was the measured variable, water with high TDS levels is likely to have high total suspended solids (TSS) as well since sewage is composed of large objects that are gradually broken down. To ensure consistency between measurements, samples for water quality properties were made at the surface of the water. This was accomplished by gradually lowering the probe until the unit display changed, indicating the probe tip was in the water, at which point the measurements were recorded. If at any time the probe slipped, it was removed and rinsed, and the measurement was repeated. Because water property measurements were taken at the water's surface, a possible explanation is that high TDS levels corresponded with high TSS and floating sewage which covers the surface and interferes with the larval development by impeding the larva from accessing the water surface for breathing. This would be a mechanism similar to polystyrene beads in pit latrines.[129] More likely though, is that *Ae. aegypti* larvae cannot develop or survive in the highly polluted waters of some septic tanks.[165]

The reason behind the positive association between mean pH and adult presence is unclear. Water pH is not considered a limiting factor for *Ae. aegypti*, and high larval survival rates are reported at ranges well outside the values found in this study.[76,166] It is possible that pH is a marker for another yet undetermined factor. This may also be the case for the negative relationship between septic water source and adult counts. When septic tanks receive water from the kitchen or shower there may be residual chemicals (e.g. soaps) which could have some adult repellency or inhibit larval respiration by altering the water surface tension.[162]

The inverse relationship between mosquito counts and the number of occupants may be due to the increased toilet usage which results in more frequent disruptions of the surface water in the septic tank. This in turn could interfere with the immature insect's ability to maintain contact with the surface, especially during vulnerable times such as pupation, and possibly result in its drowning.[162] Another possible explanation is that more people in a home results in a greater generation of fecal-waste and higher TDS values.

Limitations of this study: While the results of this study indicate that *Ae. aegypti* larvae can be found in septic tanks, they could not be used to reliably develop models for predicting presence and counts. Although several variables were significantly associated with mosquito presence and abundance, they did not adequately explain the variation among trap adult and larval counts. In a drinking well or cistern, the total larvae in a well can be predicted based on the well diameter and trap catch.[106] Unfortunately, the raw sewage in septic tanks can obstruct the funnel and prevent larvae from entering the trap. This in turn makes it difficult to accurately develop models for predicting the presence or total larval population per septic tank and is a possible explanation for the high unexplained variability in the models. Another factor which may have affected the larval traps was the insecticide treatment. Although the septic tanks were larger than the container originally tested, the insecticide may still have killed a sizable portion of the larvae which would have negatively affected trap performance. Additional studies are needed to further determine the factors that support larval development in septic tanks.

It is also difficult to determine how many of the immature stages of *Ae. aegypti* would successfully mature to adults and therefore assess the overall mosquito productivity of each septic tank. Even if it were possible to accurately determine the total number of larvae in a septic tank, larval indices do not correspond well with the number of adult mosquitoes or burden of disease.[112,167] Additionally, most of the larvae in a trap, especially in traps with high larval counts, were often dead. While this was most likely a result of oxygen depletion in the small trap reservoir, and not related to water properties of the septic tank, it does further complicate interpretation of the results.

		Response (%)
Number of occupants	1	12 (13)
	2	27 (28)
	3	19 (20)
	4	20 (21)
	5	10 (10)
	Over 5	8 (8)
Shower or bath	Shower	91 (95)
	Both	4 (4)
	No answer	1 (1)
Showers per day	Once	11 (11)
	Twice	61 (64)
	Three or more	22 (23)
	No answer	1 (1)
Shower length	5-10 min	30 (31)
	Over 10 min	65 (68)
	No answer	1 (1)
Water source	Private well	66 (69)
	Public	28 (29)
	Unsure	2 (2)
Washing machine	Yes	91 (95)
	No	5 (5)
Frequency of use	Monthly	1 (1)
	Weekly	31 (34)
	2 -3 times / week	48 (53)
	Daily	1 (1)
	More than once / day	10 (11)
Septic water source	Toilet only	53 (55)
	Toilet and shower	23 (24)
	Bathroom and kitchen	14 (15)
	Kitchen only	1 (1)
	Bath, kitchen & washing machine	5 (5)
Septic service	Monthly	1 (1)
	3-4 times / year	7 (7)
	2 times / year	9 (9)
	Once a year	10 (10)
	More than a year	31 (32)
	Unknown	38 (40)
Septic tank size	Unknown	93 (97)
	Less 7500 L	3 (3)
Construction material	Plastic	1 (1)
	Concrete & steel	6 (6)
	Concrete only	86 (90)
	Unknown	3 (3)

Table 4. Summary of water usage and maintenance of septic tanks in Playa/Playita, Puerto Rico, between February and April 2008.

Surface area (m^2)	Mean (SD)	6.64 (3.82)
	Median	6.00
	Range	1.43 – 34
Distance to house (m)	Mean (SD)	3.87 (3.47)
	Median	3.20
	Range	0 -18.9
Vent pipe length (m)	Mean (SD)	5.21 (4.04)
	Median	4.30
	Range	0.1 – 18.2
Opening distance from wall (m)	Mean (SD)	0.37 (0.23)
	Median	0.30
	Range	0 – 1.3
Above ground height (m)	Mean (SD)	0.25 (0.20)
	Median	0.2
	Range	0 – 1
Screened Vent Pipe (percent of total)	Yes	53 (88.33%)
	No	7 (11.67%)
Cracked (percent of total)	Yes	54 (56.25%)
	No	42 (43.75%)
Sun exposure	Mean (SD)	48 (38)
(percent of tank exposed to 8 hours of direct sunlight)	Median	50
	Range	0 – 100
Opening coverage (percent of total)	Open	8 (8.33%)
	Covered	60 (62.50%)
	Capped	28 (46.67%)
pH	Mean (SD)	7.56 (0.47)
	Median	7.65
	Range	5.2 – 8.3
Temperature (°C)	Mean (SD)	26.83 (1.59)
	Median	26.85
	Range	19 – 32
TDS (ppt)	Mean (SD)	1.356 (0.667)
	Median	1.127
	Range	0.600 – 3.985
Conductivity (µS)	Mean (SD)	2.656 (1.240)
	Median	2.255
	Range	0.800 – 7.320

Table 5. Summary of water properties and local environment of septic tanks in Playa/Playita, Puerto Rico between February and April 2008.

Notes:

TDS = total dissolved solids

ppt = parts per thousand

µS = microSiemens

SD = standard deviation

	Mean daily trap count among all tanks	95% CI	Present (%)	Mean daily trap count among positive tanks	95% CI	Min.	Max.
Aedes aegypti							
Larvae (n = 89)	1.94	-0.29 – 4.16	16 (17.98)	10.28	-2.34 – 22.90	0.25	87
Adults (n = 93)	4.34	1.76 – 6.93	46 (49.46)	8.67	3.66 – 13.67	0.33	85
Culex							
Larvae (n = 89)	101.55	52.39 – 150.71	66 (74.16)	129.62	64.90 – 194.34	0.25	1517
Adults (n = 93)	150.46	88.95 – 211.96	90 (96.77)	155.48	92.15 – 218.80	0.33	2268

Table 6. Results of floating funnel and adult emergence trappings of septic tanks in Playa/Playita, Puerto Rico between February and April 2008.
Note: CI = Confidence Interval

	Univariate		Multivariate	
	OR	p value	OR	p value
***Aedes* larvae**				
Cracked	3.68	0.058	3.95	0.053
Opening status (open, covered,	2.93	0.045	3.27	0.048
***Aedes* adults**				
Cracked	2.24	0.059	-	0.1357
Uncapped opening	2.23	0.085	-	0.1040
pH	2.49	0.071	3.69	0.028
Number of occupants	0.67	0.009	0.62	0.004
***Culex* larvae**				
TDS	0.24	0.001	-	-
Conductivity	0.999	0.005	-	-
Public water source	3.90	0.042	N/A	N/A

Table 7. Significant associations between septic tank environmental and water quality variables and mosquito presence in septic tanks in Playa/Playita, Puerto Rico between February and April 2008.
Note: OR = Odds Ratio

	Univariate		Multivariate	
	IRR	p value	IRR	p value
***Aedes* larvae**				
Surface area	2.09	0.002	1.54	0.058
Above ground height	0.001	0.006	-	0.7220
Uncapped opening	11.31	0.042	29.97	0.037
TDS	0.1	0.043	0.06	0.018
Conductivity	0.33	0.061	-	-
Number of occupants	0.18	0.008	0.27	0.012
Tank age	2.59	0.086	-	0.4073
***Aedes* adults**				
Cracked	2.91	0.054	-	0.1322
TDS	0.19	0.000	-	0.2449
Conductivity	0.999	0.005	-	0.2152
Number of occupants	0.48	0.000	0.47	0.000
Washing-machine use	0.43	0.009	-	0.4248
Septic source	0.67	0.096	0.69	0.088
***Culex* larvae**				
Cracked	2.70	0.056	-	-
TDS	0.36	0.061	-	-
***Culex* adults**				
Opening status	0.51	0.075	-	-
Shower length	0.75	0.038	-	-

Table 8. Significant associations between the septic tank environmental and water quality variables and mosquito counts in septic tanks in Playa/Playita, Puerto Rico between February and April 2008.

Note:

IRR = Incidence Rate Ratio

TDS = total dissolved solids

		Aedes aegypti adult presence		
		Yes	No	
Aedes aegypti larval presence	Yes	13 (7.8)	2 (7.2)	15 (15)
	No	32 (37.2)	39 (33.8)	71 (71)
		41 (41)	45(45)	86

Fisher's exact test = 0.004

Table 9. Observed and (expected) numbers of septic tanks positive for *Aedes aegypti* adult and larval mosquito presence in Playa/Playita, Puerto Rico between February and April 2008, with the results of Fisher's exact test for independence.

Chapter 5 - Control of *Aedes aegypti* Mosquitoes in Septic Tanks

Results

Adult emergence trap results are summarized in Table 10. Traps could not be placed during the month of August due to scheduling difficulties. Additionally, four septic tanks were flooded during September. An emergence trap was not used on the flooded septic tanks. A Wilcoxon rank-sum test was used to assess the two groups (2,2 – dichlorvos treated and control septic tanks) at baseline and the monthly change in trap counts relative to baseline values.

At baseline, (e.g. April) *Ae. aegypti* adults were recovered from three of the 13 untreated tanks and the median number of *Ae. aegypti* adults per septic tanks was zero (range 0 – 5). *Aedes aegypti* adults were recovered from five of the 12 pre-treated septic tanks and the median number of *Ae. aegypti* adults per septic tank was again zero (range 0 – 12). There was not a statistically significant difference between the two groups at generally accepted levels ($z = 0.92$, $p = 0.36$). When considering the total trap count, both *Ae. aegypti* and *Cx. quinquefasciatus* adult mosquitoes were recovered from 13 of the 13 untreated tanks (median 10, range 1 – 421) and 12 of the 12 treated septic tanks (median 24.5, range 3 – 662), prior to introduction of the 2,2 dichlorvos impregnated strip. There was not a statistically significant difference between the two populations using generally accepted values (Wilcoxon rank sum, $z = 1.44$, $p > 0.15$). Medians are reported instead of means due to the small sample size and highly skewed data (standard deviation ≥ twice the mean).

One month after adding the 2,2 dichlorvos impregnated strip there was a statistically significant difference between treated and untreated septic tanks, with regard to the total number of mosquitoes (Wilcoxon rank sum, $z = -2.53$, $p < 0.0115$). Adults were recovered from 12 of the 13 untreated tanks (median 40, range 0 – 1336) and 11 of the 12 treated tanks (median 6, range 0 – 126). *Aedes aegypti* were recovered from six of the 13 untreated tanks (median 0, range 0 – 10) and two of the 12 treated tanks (median 0, range 0 -2). This difference was not significant at generally accepted levels ($z = -1.93$, $p > 0.053$). There was no significant difference in trap counts, for both *Ae. aegypti* and total mosquitoes, between the two groups during any of the other months ($-1.52 < z < 0.22$, $p > 0.13$).

Discussion

Since there was not a significant difference in trap counts between the two groups at baseline (e.g. April), the subsequent monthly comparisons between the two groups could have been made using the absolute trap counts instead of using the monthly change in trap counts from baseline values. It must be noted that when using this method, there was no significant difference in mosquito trap counts between the two groups during any month; although, although the difference in both *Ae. aegypti* and total trap counts between the two groups did approach significance during May ($z = -1.6$, $p = 0.1$). After consulting with the university biostatistics department, the monthly change in septic tank trap counts from baseline values was still used when comparing the two groups for three reasons. First, it was theorized the trapped mosquito populations in each group may not have come from a normal distribution and using the monthly change in trap counts could help control for some of the variance between septic tank mosquito populations. Second, the initial decision to use the monthly change from baseline method was made prior to examining the baseline values, so that the method selection was not affected by the results of the analysis. Finally, although the two methods have differing statistical significance at one month post treatment, they both result in the same conclusion regarding the use of 2,2 dichlorvos impregnated strips for controlling mosquito populations in septic tanks.

These results do not support the use of the 2,2 dichlorvos impregnated strip for controlling mosquito productivity in septic tanks, at least not for periods longer than one month. Although the manufacturer states the strips control insects for up to four months, their effectiveness was limited to only one month in the Las Mareas septic tanks. Moreover, the strip's effectiveness at one month was largely due to a relative reduction in the number of *Cx. quinquefasciatus* mosquitoes as the reduction in *Ae. aegypti* mosquitoes was not statistically significant. The small sample size of this may have contributed to an inability to detect a significant difference in *Ae. aegypti* counts at one month; but this does not change the conclusion that the impregnated strips are not suitable for long term control of mosquito productivity in septic tanks. While the individual cost of the strip is relatively low, approximately US$ 5, given their short duration of effectiveness the annual cost is US$ 60 per house. Even in a small community like Las Mareas, it would still cost nearly US$ 20,000 per year to treat every septic tank which is cost prohibitive.

A possible explanation for the strips' short duration of control is the unique microenvironment of the septic tank. While the impregnated strip is water resistant, 2,2-dichlorovinyl dimethyl phosphate rapidly hydrolyses with moisture.[168] Septic tanks are quite humid and it was not unusual for emergence traps to have condensation formation. In a closed septic tank it is likely that considerable moisture may have accumulated on the surface of the strip which could negatively impact its performance. Another factor which could have diminished the strips' performance was flooding of the septic tanks which occurred in four of the treated tanks. This is especially problematic in coastal and low-lying communities like Las Mareas where water can leak into the septic tanks when the ground becomes overly saturated from rainfall or tidal changes.[169]

2,2 Dichlorvos Strip	April[1]		May		June		July		September	
	Aedes	Total[2]	Aedes	Total	Aedes	Total	Aedes	Total	Aedes	Total
No	0	32	1	280	0	0	0	26	0	4
No	0	24	1	40	0	54	0	0	0	60
No	0	2	10	53	0	0	0	22	1	31
No	0	5	7	9	0	5	0	12	4	30
No	0	421	0	1336	0	0	0	0	0	0
No	1	6	0	87	0	21	0	0	0	25
No	3	3	7	12	0	0	2	2	117	155
No	0	7	0	40	0	23	0	0	0	35
No	0	12	0	25	0	138	0	118	0	813
No	0	12	0	70	0	16	0	148	0	26
No	5	55	1	9	17	112	44	103	168	168
No	0	1	0	0	0	0	1	1	0	0
No	0	10	0	11	0	0	0	0	0	5
Median	0	10	0	40	0	5	0	2	0	30
Yes	4	467	0	3	0	38	0	5	-	-
Yes	1	26	0	37	0	0	0	0	50	381
Yes	1	9	0	3	0	3	1	1	0	22
Yes	0	103	0	4	0	10	0	2	-	-
Yes	0	48	0	0	0	0	0	4	26	48
Yes	0	3	0	70	2	419	0	130	-	-
Yes	0	5	0	4	3	99	0	1	5	20
Yes	0	11	0	126	0	244	0	848	4	20
Yes	12	23	1	8	0	13	1	6	0	35
Yes	0	662	0	37	0	71	0	150	-	-
Yes	0	89	2	60	0	17	1	40	10	207
Yes	2	7	0	2	2	283	0	30	0	20
Median	0	24.5	0	6	0	27.5	0	5.5	4.5	28.5

Table 10. Monthly adult mosquito emergence from septic tanks in Las Mareas, Puerto Rico between April 2008 and September 2008 in order to evaluate the effectiveness of using a 2,2 dichlorvos impregnated strip for control of adult mosquito populations.

Note:

[1] A 2,2 dichlorvos impregnated strip was added to 12 septic tanks after collection of data on April 10th, 2008. The strips were not removed until after the final emergence trapping in September.

[2] Total number adult of *Aedes aegypti* and *Culex quinquefasciatus* recovered from once monthly emergence traps placed on the septic tanks.

Chapter 6 - Use of GIS to Examine Associations Between the Incidence of Dengue and Environmental and Socioeconomic Conditions

Results

A total of 1,065 case submissions were confirmed by the CDC laboratory between March 2003 and April 2008 in the seven municipalities of Cayey, Coamo, Guayama, Humacao, Juana Diaz, Ponce, and Santa Isabel. There was insufficient information to georeference 166 of the cases. Additionally, cases from the municipality of Juana Diaz were excluded due to incomplete reporting, leaving a total of 862 cases available for analysis. Of these, 136 cases were reported during the dry season (December- April). When monthly case totals were plotted against average rainfall, there was a delay of approximately one month between the start of the rainy season in May and the increase of dengue incidence in June (Figure 8Figure 8).

There were 263 block groups within the area of investigation, excluding the 33 block groups in the municipality of Juana Diaz. The total population was 396,433, excluding 50,531 persons living in Juana Diaz. The total person-time was 2,048,288 person-years. Total person-time was calculated by multiplying the population by the length of the observation period which was approximately 5.2 years. The total incidence rate for the five year period was 4 cases per 10,000 person-years. Block group mean values are summarized in Table 11.

During the period of March 2003 - April 2008, the incidence of dengue was negatively associated with elevation and distance to the hospital and positively associated with income (Table 12). After adjusting for the effects of the other variables, only a decrease in elevation was significantly associated with the incidence of dengue. When the analysis was limited to the dry season, the incidence of dengue was positively associated with income and the number of sewerage pixels in a block group. In the rainy season dengue incidence was positively associated with income and negatively associated with elevation and distance to the hospital. After adjusting for the other variables, dengue was negatively associated with elevation and the number of sewerage pixels. Although several variables were significantly associated with dengue incidence, I could not develop a satisfactory model to explain the incidence of dengue among block groups in southeastern Puerto Rico. In particular, the use of septic tanks (as estimated by the decreased presence of sewerage) was not a simple predictor of increased dengue incidence.

Discussion

The one month delay between the start of the rainy season and a corresponding increase in the incidence of dengue is likely a result of the time needed for mosquitoes to develop in the new, rain-filled environment, and become infective. A similar finding was also reported in Barbados and Brazil.[152,170] Temperatures are also warmer during the rainy season which shortens mosquito development time and the extrinsic incubation period. The inverse association between dengue incidence and elevation was likely a result of temperature differences as higher elevations are generally cooler.

The positive association between income and dengue incidence was unexpected. Prior studies in Puerto Rico, and elsewhere, have found an inverse relationship between income (socioeconomic status) and dengue.[52,147,171,172] Possible explanations for this negative association include an inability to afford air-conditioning or screening of windows, the presence of water storage containers in response to variable water supplies, and the general association of poverty as a proxy for many disease risk factors. However, several other studies have found either a positive relationship or no association between income and the incidence of dengue.[173-176] A possible explanation is that wealthier patients are more likely to be able to afford medical treatment and are therefore more likely to seek medical care and be seen by a physician. Given the inconsistent and varied association between income and dengue incidence, careful consideration of this variable must be made before it is included, or excluded, in future models. Further study is needed to fully evaluate the association between dengue and income status.

The possible association between septic tanks, as a result of *Ae. aegypti* presence and abundance, and dengue transmission requires additional study. I assumed communities without public sewerage sytems used septic tanks and *Ae. aegypti* productivity in those septic tanks could sustain dengue transmission when traditional surface habitats were unavailable (e.g. during the dry season). Had all parts of this assumption been true, I would have expected a negative association between the number of sewerage pixels in a block group and dengue incidence. A negative association was noted during the rainy season, but a positive association was seen during the dry season. During the rainy season increased precipitation could dilute septic tank waters, thereby making them more attractive for oviposition. It is also possible that the mosquito is developing, or adults are harboring, in the sewers. A survey in Cali, Columbia found 3.2 times more larvae and 6.7 times more pupae in sewers than in the nearby indoor containers.[177] Unfortunately, the report was limited and did not specify time of year, season, or where in the sewers the larvae and pupae were found, making it difficult to make additional conclusions. It is also unknown whether *Ae. aegypti* larvae are present in Puerto Rican sewers. The analysis at present fails to identify the presence of septic tanks, or sewerage, as a reliable predictor of dengue transmission. Seasonal differences in the contribution of these sources to overall vector populations may occur but further studies are needed to investigate the role of septic tanks in disease transmission.

I was unable to develop a model which could predict the incidence of dengue in southeast Puerto Rico during the time period between March 2003 and April 2008. While several individual variables were significantly associated with the incidence of dengue during certain time periods, only income and elevation were significantly associated during all times of the year, and no variable was consistently significant after adjusting for possible confounding. Furthermore, none of the variables could satisfactorily explain the variation in incidence between block groups.

<u>Study Limitations:</u> This study had several potential limitations which may have negatively impacted the observed results. First, underreporting of cases may have decreased the sensitivity of the dengue surveillance system. Dengue surveillance in Puerto Rico is a passive system which relies on attending physicians for serum samples and reporting. Because there is not a specific treatment for dengue, only supportive care, physicians may find it impractical to submit every sample for testing once a diagnosis of dengue has been established in the area.

Another limitation was our ability to identify the presence of septic tanks. I used the absence of sewerage lines as a proxy for the presence and usage of septic tanks, but communities can have a working sewerage system and still use septic tanks. The community of Playa/Playita in the municipality of Salinas had a sewerage system installed in 2006 but only 23.36% of surveyed homes were connected to the system two years later. Finally, in an ecological study it is difficult to identify and control for confounders and individual risk factors which could affect our results. The collection and analysis of data at the block group level may have resulted in the omittance of other environmental variables which were significantly associated with the incidence of dengue.

		Mean	Standard deviation
Total block groups	263		
Total population	396,433		
Total person-years	2,048,288		
Area (km2)		4.9	8.6
Average household size		3.1	0.4
Population density (per km2)		2443	2789
Distance to hospital (km)		4.5	3.7
Elevation (m above sea level)		119	160
Households (per block group)		482	223
Annual income (U.S. Dollars)		14,290	8,228
Mean population		1510	752
Sewerage pixels		2244	3666
Urban percentage		93	21

Table 11. Summary of U.S. Census 2000 block group data for the municipalities of Cayey, Coamo, Guayama, Humacao, Ponce, and Santa Isabel, southeast Puerto Rico.

	IRR	95% CI	Adjusted IRR	95% CI
Total period				
Average household Size	1.16		1.23	
Population density	1.00		1.00	
Distance to hospital	.96*	.93 - .99	.98	
Elevation[1]	.89†	.83 - .95	.88†	.80 - .97
Households	1.00		1.00	
Annual income[2]	1.02*	1.00 - 1.02	1.01	
Sewerage pixels[3]	1.00		1.01	
Urban percentage	1.30		.999	
Wet season				
Average household size	1.21		1.31	
Population density	1.00		1.00	
Distance to hospital	.95†	.92 - .98	.97	
Elevation[1]	.89**	.82 - .95	.87†	.79 - .96
Households	1.00		1.00	
Annual income[2]	1.01†	1.00 - 1.03	1.00	
Sewerage pixels[3]	.998		.997*	.994 - .999
Urban percentage	1.29		.60	
Dry season				
Average household size	.89		83	
Population density	1.00		1.00	
Distance to hospital	.995		1.02	
Elevation[1]	.88*		.91	
Households	1.00		1.00	
Annual income[2]	1.04†	1.01 - 1.06	1.03†	1.01 - 1.06
Sewerage pixels[3]	1.01*	1.00 - 1.01	1.005*	1.00 - 1.01
Urban percentage	1.48		1.43	

Table 12. Association between dengue incidence rate ratios during the time period of March 2003 – April 2008 in the municipalities of Cayey, Coamo, Guayama, Humacao, Ponce, and Santa Isabel, southeast Puerto Rico, and U.S. Census 2000 block group data.

Notes:

IRR = Incidence Rate Ratio

† $p < .01$

*** $p < .05$**

CI = Confidence Interval

[1] = IRR per 100 meter increase

[2] **= IRR per $1,000 increase**
[3] **= IRR per 1,000 pixel increase**

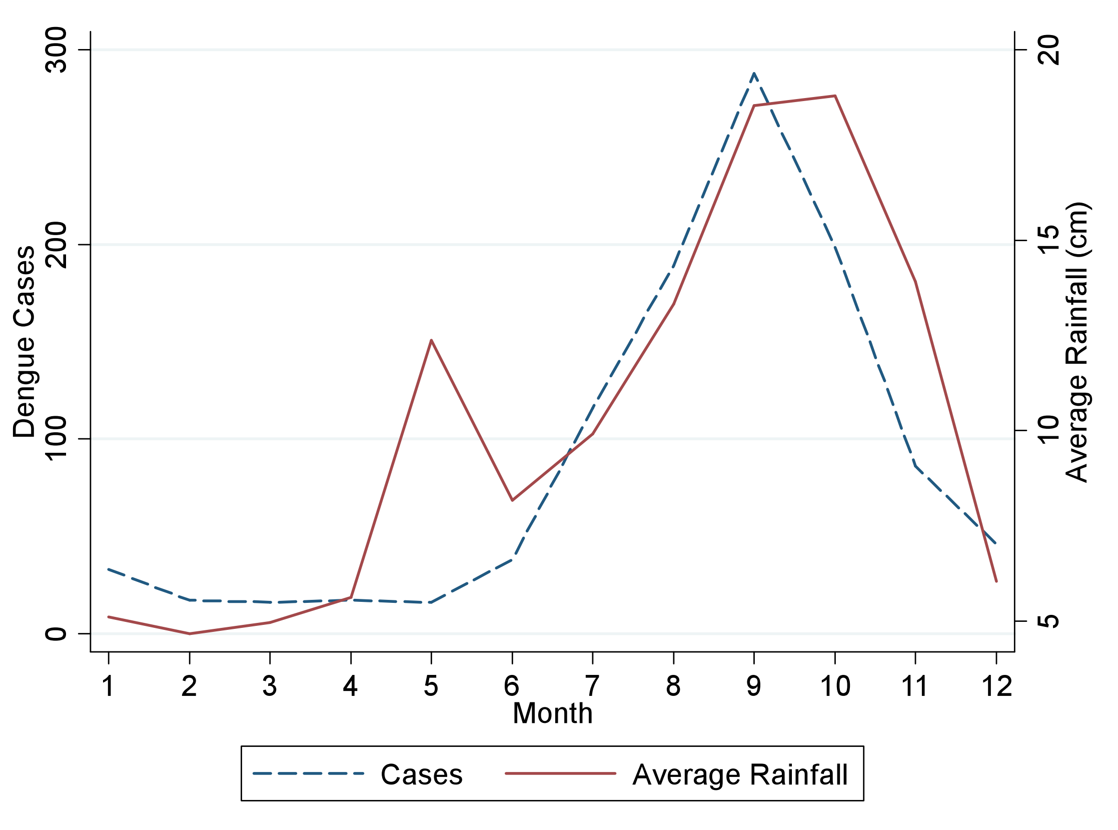

Figure 8. Dengue incidence and average centimeters of rainfall in the municipalities of Cayey, Coamo, Guayama, Humacao, Ponce, and Santa Isabel (southeast Puerto Rico) by month, between March 2003 and April 2008.

Chapter 7 - Conclusions

Public Health Significance

This study required the modification of a previously validated funnel trap for use in standard underground septic tanks in Puerto Rico.[106] Although the miniaturized Vietrap has a noticeably smaller opening diameter than the original model, it proved to be an effective tool for detecting larval presence in septic tanks with narrow access port openings. As previously discussed, it is likely that the effect of the narrower opening on trap performance was likely offset by the shallower draft of the miniaturized model. The shallower draft of the miniaturized Vietrap meant that *Ae. aegypti* larvae need not dive as deep in order to become captured by the floating funnel trap.

This study strongly suggests that *Ae. aegypti* larvae and pupae are present in Puerto Rican septic tanks containing raw sewage. These findings are inconsistent with early reports where the mosquito larvae were found only in clean, unpolluted water, and never in water containing fecal waste.[162,178-180] More recent research suggests the species may have adapted to the unique, and highly polluted environment, of septic tanks.[78,81,82,181,182] The findings from this study are consistent with these later published reports. Although the previous studies reported *Ae. aegypti* larval presence in septic tank effluent discharges and in septic tanks in general, this is the first reported study to document *Ae. aegypti* larvae and pupae in septic tanks containing raw sewage in the Caribbean.

Aedes aegypti presence in septic tanks may have a significant impact on the transmission of dengue fever. This study reported a mean *Ae. aegypti* emergence trap count of 8.67 adults per day from the septic tanks. Large numbers of *Ae. aegypti* adults were also collected from emergence traps on septic tanks in the previous CDC study in Playa/Playita.[82] Setting aside the issue of larval development, these results suggest septic tanks are important sites of adult mosquito harborage. The relative importance of this harborage is underscored by the behavior of the female *Ae. aegypti* mosquito. Whereas the male mosquito spends a considerable amount of time in exposed, indoor and outdoor positions, the female adult is generally found in heavily shaded and protected indoor areas; or as in this case, a septic tank. This behavioral difference is most noticeable with regards to adult control measures and the use of ULV drift sprays.

ULV applications rely on wind and air movement for dispersion and subsequent contact with adult mosquitoes. As adult males are generally found in more vulnerable locations, they will often have a higher mortality rate, approaching 100 percent, following ULV application.[21,117] When caged females are placed in similar exposed locations, 100 percent mortality rates have been observed; however, when the cages were placed in the more traditional adult female resting locations, mortality rates were between 0 and 25 percent.[21] This is likely a result of the limited air flow in these protected areas which reduces the likelihood of the female mosquito coming in contact with the insecticide. Since eggs, pupae and larvae are also unaffected by ULV sprays, the adult population rapidly returns to normal after ULV applications. The protection provided by the septic tank to the resting adults inside would likely shield the mosquitoes from ULV applications, which would lessen the insecticide’s effectiveness to reduce the adult population within a community.

Returning to the issue of possible larval and pupal development within the polluted waters, septic tanks have the potential to produce significant numbers of adult *Ae. aegypti* mosquitoes. As previously mentioned, studies have found that certain artificial containers may be more productive than other containers within a geographic location.[84,85,112] Equally important is the concept that the most abundant container may not be the most prolific producer of mosquito pupae, and therefore, adult mosquitoes. For instance, the survey in Salinas, Puerto Rico found that while animal drinking pans were relatively common in the community, ~140, they produced very few female *Ae. aegypti* pupae, between 50 and 100. Conversely, plastic covers were relatively rare, ~25, but very productive in terms of female *Ae. aegypti* pupae, ~450. The study also concluded that while removing the most common water containers could result in a 60 – 80% reduction in the number of pupae, it may not be physically or economically feasible. Instead, it recommended that pupal surveys be used to identify the most productive, versus the most prolific, containers and that source reduction efforts be directed towards these containers.

Although the number of septic tanks, relative to the total number of containers, is likely to decrease as the community population increases, they still have the potential to be significant sources of *Ae. aegypti* productivity. The relative decrease in septic tank frequency is due to an increasing probability of having a sewerage system with larger communities. However, aside from the daily influx of wastewater from the home, septic tanks remain relatively undisturbed for long periods of time. Only one of the homes surveyed in this study reported a monthly servicing (pumping out) of the septic tank; and over 80% of the homes reported they serviced the septic tank no more than annually, if at all. *Aedes aegypti* larvae have been noted to completely develop from egg to adult in 9.7 ±0.2 days at 25 ºC, a temperature which was slightly below the mean of 26.8 ºC reported in this study.[68] If the larvae and pupae are capable of developing in septic tanks, this suggests septic tanks could produce significant numbers of adult mosquitoes in between servicing appointments, especially if that service is provided only once a year.

The relative importance of septic tanks as larval and pupal aquatic habitats could also increase during the winter months (i.e. dry season) when other surface containers are unavailable due to the lack of rainfall in Puerto Rico. The previously mentioned CDC study noted that the number of resting adult *Ae. aegypti* mosquitoes per room was significantly lower in the nearby community of Coqui than it was in Playa/Playita, following a surface container source reduction intervention.[82] Both communities are located in southeast Puerto Rico, about six miles apart, and were of approximately similar size and composition, except for the fact that Coqui utilized a fully functional sewerage system, while Playa/Playita did not. It was hypothesized that *Ae. aegypti* productivity in the septic tanks was likely responsible for failed intervention in Playa/Playita, and that in other communities with septic tanks, this productivity may be responsible for continued transmission of dengue during periods when environmental conditions do not support mosquito development in the more traditional surface containers. Assuming this hypothesis is true, controlling *Ae. aegypti* productivity in septic tanks may help reduce ‘overwintering’ of dengue with the goal of reducing the overall burden of disease the following rainy season.

Controlling mosquito productivity in septic tanks may also be a cost effective means of significantly reducing the number of pupae per person to levels which do not support disease transmission.[32] During the rainy season, there are numerous water-filled containers which are suitable for larval development, making source reduction efforts costly and difficult. However, in the dry season, the number of suitable surface containers is greatly reduced. Moreover, while most houses can have multiple water filled buckets, pails, or discarded trash items, they are unlikely to have more than one septic tank. If septic tanks are indeed significant sources of mosquito productivity, the reduced number of them, relative to the total number of containers, makes them ideal targets for source reduction as efforts directly against them are likely to significantly reduce the number of pupae per person with a minimal expenditure of time, effort, and money.

This study found *Ae. aegypti* larvae in 18% of the sampled tanks. The mean daily trap count for many individual septic tanks was 0.25 larvae per day (one larva in four days of trapping); however, at least one tank had a mean daily trap count of 86.75, and the mean trap count among all positive septic tanks was 10.28 larvae per day (minimum 0.25, maximum 86.75). Applying the regression plot data from Chapter 3, $Y = 0.0001X + 0.02$ (Y = larval density and X = trap count), the result is a projected mean population density of 0.021 larvae per cm^2. Using the mean surface area for septic tanks from Chapter 4 of 6.64 m^2, this gives a mean daily *Ae. aegypti* population of 1,394 larvae per septic tank. If we repeat the above calculations for the septic tank which had a mean daily trap count of 86.75 and a surface area of 10.23 m^2, the estimated larval density and daily larval population are 0.029 larvae per cm^2 and 2,938 larvae, respectively. Unfortunately, as discussed later under the limitations section, it is difficult to relate these larval numbers to the incidence of dengue or burden of disease. Nonetheless, the high larval populations in septic tanks have the potential to develop into large numbers of adult mosquitoes which can significantly impact dengue transmission in communities with septic tanks, especially during the dry season as discussed in the preceding paragraphs.

Unfortunately, while controlling *Ae. aegypti* productivity in septic tanks within Puerto Rico may help reduce the burden of disease, the results of this study do not support using 2,2 dichlorvos impregnated strips for controlling *Ae. aegypti* mosquitoes in septic tanks. Although the strips reduced the total number of captured mosquitoes one month post application, they did not significantly reduce the number of *Ae. aegypti* mosquitoes, and they were ineffective at reducing overall numbers for periods longer than one month. As previously discussed, a possible explanation for the strips' inadequate control is the unique micro-environment of septic tanks which may have rapidly degraded the effectiveness of the strips. Unfortunately, the challenges caused by this micro-environment are likely to impact other control methods as well. Although larvicides were successfully used to control *Culex pipiens* (L.) in Turkish septic tanks, their effectiveness was affected by the daily influx of water and organic matter.[183] A primary means for the degradation of many pesticides is through the mechanism of hydrolysis.[184] By nature of their intended purpose, septic tanks are very humid environments, and this moisture may rapidly degrade the pesticides.[185-188] The degradation rate may also be more rapid as the ambient temperature increases. At low temperatures (5 ºC) the half-life of pesticide florasulam can be 85 days, depending on soil type. Under similar soil conditions, but at a higher temperature (20 °C), the half-life is 8 days. At temperatures like those found in a septic tank (25 ºC), the breakdown can occur in as little as 1 day, depending on soil type. Even if a pesticide is designed to withstand the temperature and moisture levels in a septic tank, high levels of suspended solids can bind with the pesticide and reduce the bioavailability of the chemical to sub-lethal levels.[189] Another concern regarding the use of pesticides is the possibility that larvicides may gradually leach out of the septic tanks. Over 6% of surveyed homes had a dry septic tank, 32% had not serviced their septic tank in over a year, and 40% of homes could not remember the last time they serviced the septic tank. Since only one septic tank was found overflowing with sewage, it is likely that the contents of the rest of the septic tanks were slowly seeping into the ground. Not only would leaching of the insecticides reduce their effectiveness, but it could endanger the community drinking water as many wells were located near a septic tank.[190]

The two biggest challenges though concerning the use of insecticides are the issues of cost and manpower. As discussed earlier, the chemical structure of the pesticide is likely to rapidly degrade or otherwise become ineffective due to binding with the organic debris in the microenvironment of the septic tank. Controlling mosquito productivity in septic tanks with insecticides would thus require multiple treatments throughout the year which would be very manpower and cost intensive.

A larvicidal study in Oman can be used to approximate the costs of such a program.[191] The study used temephos, a common insecticide for treating surface containers, and one of the pesticides that was used to control *Cx. pipiens* in septic tanks in the Turkish study.[183] Using the recommended application dosage and frequency (1.0

ppm, fortnightly), the total cost of 14 treatments for a 3,538 m^2 area was US$ 971. For comparison, assuming only 50% of the 1,013 homes in Playa/Playita still used a septic tank and the mean septic tank surface area is 6.64 m^2, the total surface area is 3,363 m^2. The total cost in the Oman study was also determined using a daily wage of only US$ 13 for the workers which is well below the U.S. minimum wage. The minimum wage in Puerto Rico varies by industry, but the lowest hourly rate is US$ 4.10.[192] Assuming an eight hour work-day, the daily wage of US$ 32.80 per worker is more than twice what the Oman study reported. The Oman study also found that fortnightly applications of 1 ppm temephos were generally ineffective and better control was obtained using 0.5 ppm temephos weekly, although this method cost almost twice as much (US$ 1792) as the fortnightly applications. While other insecticides or methods of temephos application (e.g. non-woven sachets of granules) may last longer, require fewer applications and therefore be slightly less expensive, it is still likely the total cost of such a program will not be insignificant.[126] Given these costs, it is unlikely that the government could sustain insecticide applications for septic tanks in the small community of Playa/Playita, let alone treat the whole island in order to control the mosquitoes in septic tanks.

Rather than using chemicals, it could prove more effective to ‘mosquito proof’ the septic tanks. Although this would have a higher initial cost, it may be more cost effective in the long-term. In 2006, the CDC determined that *Ae. aegypti* was associated with open or cracked vent pipes and placed screens on several of the vent pipes within the community.[82] Two years later, over 88% of the sampled vent pipes were still screened. An additional benefit of this method of control is it is ‘bottom up’. Bottom up interventions rely on community members to implement control measures rather than using government employees or contractors.[9] Instead of having government employees apply insecticides to control the mosquitoes, the program would provide community members with the education and tools to do it themselves. Although this type of intervention strategy can require substantial time to properly educate and encourage the ‘buying-in’ of the community members, it is more likely to be sustained in the long-run due to its low cost. Another benefit of the bottom-up approach is that successful implementation will likely have a greater impact on disease transmission than government applications of insecticides. As mentioned earlier, weaknesses of ULV insecticide applications are an insect’s development of resistance, little residual activity, and little to no proven ability to effectively lower the number of adults or reduce the burden of disease.[15,117,118,193] Conversely, a successful bottom-up intervention can have a considerable impact on the adult mosquito population and the burden of disease through significant source reduction of larval and pupal habitats. Due to the large number of *Ae. aegypti* larvae and adults which were recovered from septic tanks in this study, and the previous CDC study, adding methods to mosquito proof septic tanks could help improve community based interventions for mosquito control.

This study was not able to determine whether *Ae. aegypti* presence in septic tanks is associated with dengue transmission. As discussed in Chapter 6, I used maps of sewerage lines to identify areas which did not have a sewerage system and assumed these areas therefore used septic tanks for their wastewater. If *Ae. aegypti* presence in septic tanks was associated with dengue transmission, those areas with septic tanks (e.g. without a sewerage system) would have an elevated incidence of dengue, after adjusting for other variables which were known to be associated with dengue transmission. The positive association between sewerage and dengue incidence during the dry season supports the null hypothesis that septic tanks are not associated with dengue transmission.

This study was also unable to explain the incidence of dengue using the environmental and ecological data available from the U.S. Census 2000 and regional weather reports. As will be discussed under the limitations section, the problems of underreporting and low sensitivity, as well as the presence of unidentified and uncontrolled confounders, may have contributed to the study's inability to explain the differences in the incidence of dengue between block groups. However, while no variable adequately explained the variation in dengue incidence between block groups, individual variables (e.g. lower elevations, increased distance to hospital, and higher incomes) were significantly associated with an increased incidence of dengue, which suggests GIS could be useful for predicting dengue incidence in Puerto Rico with further refinement.

Limitations

This study had several limitations. The unknown effect of septic tank environmental conditions on the miniaturized Vietrap's performance was one of the limitations. The floating funnel trap in this study was tested under laboratory conditions due to concerns over safety and aesthetics. Aside from occasional shadowing from laboratory personnel, the larvae in the pools were generally undisturbed. On the other hand, wastewater discharge into a septic tank would disturb the surface water and the larvae, which would most likely cause them to dive. Any increase in larval diving frequency should increase the probability of a larva diving below the funnel mouth opening and entering the trap. However, the increase in trap performance due to wastewater disturbance may be offset by the presence of floating sewage within the septic tank. Under laboratory conditions, the funnel mouth was unobstructed and larval movement into the trap reservoir was unimpeded. In septic tanks, floating sewage could block the funnel opening and prevent larvae from entering the reservoir which would decrease the trap's performance. Sewage may have also restricted trap movement, limiting the trap to a small corner of the septic tank. The restricted movement would prevent the trap from sampling clusters of larvae in other portions of the tank and lower trap performance. Conversely, if the larvae clustered in the restricted area of the floating funnel trap, this would increase trap performance. Although clustering of larvae under the trap was possible, it most likely did not significantly alter the results due to the repeated trappings and measurements. Larval samples were collected for four consecutive days. While a floating trap may have remained over a particular cluster of larvae for 24 hours, it is unlikely that it would have remained over a cluster of larvae all four days unless there were significant numbers of larvae in the septic tank. A final factor to consider is the issue of septic tank size. Previous work has shown that trap performance is inversely affected by the surface area of a container.[106] While the surface area of the laboratory pools (1.77 m^2) was within the septic tank surface area range reported in this study (0.3 – 33.55 m^2), it was noticeably smaller than the mean septic tank surface area (6.64 m^2).

Without testing the floating funnel trap under similar conditions, it is not possible to determine the actual effect of the septic tank environment on trap performance. As such calculated values using regression plots from controlled laboratory should be treated with caution and viewed as approximations instead of absolute values of the total larval population in a septic tank and should not be compared to predicted larval populations in other habitats (e.g. wells) where environmental conditions may be different. However, despite this limitation, these regression plots are still useful for estimating larval populations within septic tanks and comparing mosquito productivity between septic tanks.

The treatment of septic tanks with the 2,2 dichlorvos impregnated strip may have also impacted our study. The insecticide treatment and adult emergence trapping was performed as a back-up should the miniaturized floating funnel trap prove ineffective in septic tanks. In laboratory testing, 50% of larvae were killed at a three hour exposure to the strips. Although a Wilcoxon sign-rank test did not show a significant difference between floating funnel trap counts pre- and post-insecticide treatment, ($z > -1.363$, $p > 0.17$), it is still possible that larval mortality occurred as a result of the treatment which could have affected the trap results and subsequent conclusions. It is believed though that this mortality, and any effect on the results, would have been slight. First, although the exact volume of the septic tanks is unknown, their volume was considerably larger than the 125 L container used to test the strips. It is likely that pesticide concentration levels were thus lower in the septic tanks than in the 125 L container which may reduce larval mortality effects. Second, the septic tanks contained considerable organic material which could have protected the larvae and pupae by binding with the insecticide.

The study's power was less than expected and was also a limitation. The sample size needed to detect differences between septic tanks with adult or larval *Ae. aegypti* and those without *Ae. aegypti* was calculated at 100 septic tanks; however, four tanks were excluded as they were dry, leaving the study with 96 septic tanks. Additionally, the sample size calculations were made assuming *Ae. aegypti* prevalence was 36% instead of the 18% reported here. This resulted in a 23% decrease in power from 80% to 57%. Any decrease in a study's power results in an increased probability of having a Type II error, or falsely rejecting an alternative hypothesis. In this study, the decrease in power may have reduced our ability to detect significant associations and adequately explain the larval variation between septic tanks.

Power was also less than desirable in the GIS analysis. The incidence rate for dengue in our study was much lower than expected. During the period of March 2003 – April 2008, the dengue incidence was 4 cases per 10,000 person-years. A primary reason for the small incidence rate was the need to extend the sampling timeframe beyond the 2007-2008 outbreak. However, even limited to the 'epidemic' period of July 2007 – April 2008 the incidence of dengue was 1 case per 1,000 person-years which was still less than the 7 cases per 1,000 person-years that were used in the initial calculations. The initial estimate was based on results of the 1994 – 1995 Puerto Rico dengue epidemic where the total incidence was 7.01 cases per 1,000 persons, and 11.8 cases per 1,000 persons among high risk groups.[23] Had I had the expected incidence I could have detected differences between exposed and unexposed individuals where the relative risk was 1.06 or greater. Unfortunately, the lower incidence meant I could only detect differences when the relative risk was 1.23 or greater. A possible explanation for the low incidence is the previously mentioned probability of underreporting.

The lower than expected power in the GIS analysis may have contributed to the study's inability to develop a model for predicting the incidence of dengue. Although significant individual associations between dengue and several environmental and socioeconomic factors were found, none of the associations were consistent between the rainy season, dry season, and the total time period. This inconsistency prevented me from developing a reliable model to explain the incidence of dengue. Unfortunately, even when considering the individual seasons, I was still unable to develop a valid model for predicting dengue.

A concern in the GIS portion of this study was the possibility of underreporting of dengue during the study period. Excluding active hospital reporting for cases of dengue hemorrhagic fever, dengue fever surveillance in Puerto Rico is largely passive in nature. While passive surveillance is inherently less expensive than active surveillance, it has a higher risk of underreporting so it is possible to miss cases. Underreporting is especially problematic with rare diseases, or as the case may be here, with rare associations as the passive surveillance systems may not be sensitive enough to detect the disease or association.

Another factor which may have negatively impacted the study's ability to detect an association between the environmental factors and the incidence of dengue is the fact the presence of a sewerage system does not necessarily mean there is an absence of septic tanks. The community of Playa/Playita in the municipality of Salinas had a sewerage system installed in 2006 but only 23.36% of surveyed homes were connected to the system two years later. If *Ae. aegypti* presence in septic tanks is associated with dengue transmission, the presence of even a few septic tanks in a community with sewers could raise the incidence of dengue, making it difficult to detect an association between the two.

Another point to consider is the issue of confounding. The GIS study was conducted at the population level rather than at the individual level. Although I attempted to control for possible confounding by adjusting for variables which were either suspected or were previously shown to have an association with dengue transmission, it is difficult to adequately manage confounding in an ecological study. There may have been other variables (e.g. surface container abundance) which could have significantly impacted the results of this study, but were not identified. The problem of confounding is further complicated by the length of the study time period and the date of the Census data collection. The Census data was collected in 2000, the study examined dengue incidence during a five year period between 2003 and 2008, and sewerage maps were from 2007. It is possible that conditions may have changed during this time which could have impacted the study's ability to detect significant associations. For example, if a sewerage system in a block group was installed in 2007, it was considered present throughout the time period. Even if the system was installed in 2002, prior to the start of the study, it is unlikely that every household would have immediately switched over from septic tanks to sewers; rather, it would probably be a gradual process.

This study indicates that *Ae. aegypti* larvae are present in septic tanks containing raw sewage, but it has not conclusively shown that the larvae are capable of developing there. Although all stages (larvae, pupae, and adults) were recovered from the funnel traps, this does not indicate that development is occurring in septic tank waters. It is possible that development occurs in relatively clean 'pockets' of water that are located in the drainage pipe or in the tank wall above the actual septic pool and the recovered mosquitoes were actually washed into the tank via rainfall or flushing of the toilet rather than developing there. That large numbers of larvae were recovered from some septic tanks suggests this is not the case, and that development is occurring within the septic tank waters; however, this study cannot make that definitive statement. Confirmation of this hypothesis will require observation of complete larval development and emergence from septic tank waters containing raw sewage.

A final limitation of the study is the fact that larval numbers and indices like The House, Container, and Breteau do not correspond well with adult mosquito populations or the burden of disease.[112,167] Given the nature of septic tanks, it is likely that there is considerable variation in the aquatic conditions between septic tanks which could adversely affect larval survival and further complicate the issue. Only 55% of the septic tanks in this study received wastewater from just the toilet, while 24% received it from the toilet and shower, and 15% received wastewater from the bathroom and kitchen. The type and frequency of use for common household chemicals (e.g. cleaners, soaps, and shampoos) likely varies by home and in some septic tanks these chemicals could reach levels which are lethal to mosquito larvae.[194,195] Another factor which would affect larval mortality rates is the frequency of wastewater entering the tank. Frequent disruption of the surface water, as may be the case with high occupancy homes, could lead to larval drowning and death. Changes in water levels from water exiting the septic tank through subterranean cracks in the tank wall could lead to sewage concentrations which do not support larval life through their chemical action or physical blockage of the larvae’s access to the water’s surface. Larvae could also become trapped in the floating debris (e.g. toilet paper) and drown. Aquatic larvae of other species which feed on *Ae. aegypti* larvae could be present in septic tanks, though these were not assessed in this study.

While *Ae. aegypti* pupal counts from septic tanks would help to relate *Ae. aegypti* presence and abundance in septic tanks to the risk of dengue transmission, they are difficult to obtain. Pupae do not forage for food, so diving behavior is often done only in response to a perceived threat (e.g. shadowing, disruption of surface water). As such, few pupae would be captured using a passive tool like the floating funnel trap. Although a floating pupal trap has been developed, its size and the impact of sewage on trap performance make it less than ideal for use in individual septic tanks, making it difficult to determine the number of pupae per septic tank.[59] Dips and sweep nets are also impractical due to small openings, as well as the presence of floating sewage.

Despite the limitations of the larval indices, septic tanks could still produce significant numbers of adult mosquitoes. Using the previous calculation of 1396 larvae per tank, even with a 99% larval mortality rate, septic tanks could have a standing daily count of 13.96 or more pupae. Using an average household size of 3.15 persons, this would equate to 4.43 pupae per person. At 26 °C, this is theoretically high enough to result in a 10% increase in dengue seroprevalence within the community, assuming two-thirds of the population was already immune. Depending on the local population's dengue immunity and environmental conditions, mosquito productivity in septic tanks could play an important role in maintaining dengue at low levels during the dry season.

Suggestions for Future Studies

While this study indicates *Ae. aegypti* larvae are present in septic tanks containing raw sewage, further work is still needed. As mentioned in the limitations section, the larval sampling portion of this project was underpowered. Using a larger sample size to examine septic tanks for larval presence could provide more information on the associated factors and may help us develop a better model for explaining why the larvae are present in some tanks but not in others. This information could be useful for developing subsequent control strategies.

Further studies are needed to confirm whether the *Ae. aegypti* mosquito is developing in septic tanks. It may also be useful to determine if the ability to survive in septic tanks containing raw sewage is a trait unique to this region. When placed in synthetic sewage, *Ae. aegypti* larvae from various geographic areas exhibited differences in survivorship.[165] Molecular studies may also help determine whether this ability is a result of a recent genetic adaption.

A dengue seroprevalence study would help examine the potential role of septic tanks in dengue transmission. Researchers in Australia found a positive association between dengue seroprevalence and proximity to a subterranean well.[196] By identifying a community that is primarily on a sewerage system, and has relatively few active septic tanks, it may be possible to conduct a similar study in Puerto Rico in order to determine if septic tanks are associated with dengue seroprevalence.

The development of a working model for predicting dengue incidence in Puerto Rico could also prove beneficial. The dengue control program in Puerto Rico has five key components: a surveillance system, a rapid response plan for dengue outbreaks, a contingency plan to hospitalize large numbers of dengue hemorrhagic patients if necessary, education of the medical community on recognition of the early signs of dengue and proper treatment protocols, and a community-based *Ae. aegypti* control program.[9] Using GIS software to predict and analyze outbreaks of dengue can help address the first two components. In this study, the home addresses of dengue patients between March 2003 and June 2007 had to be manually retrieved from paper records by CDC personnel. As of July 2007, the patient home addresses for positive dengue case submissions are routinely entered into the electronic database. Electronic entry of the home address simplifies the georeferencing process so that the future GIS investigations could be extended to the whole island rather than limiting them to just one area or region. Collecting several years of case data and working with smaller geographic units (e.g. Census block) could both improve the analysis. This may be especially true when using weather data (e.g. rainfall, humidity, temperature). Although municipality level weather data is easier to obtain, it may not necessarily be uniform across the entire municipality due to the large geographic area of some municipalities and variations in elevation. Reporting this data at a smaller level could address some of this variation. GIS analysis of the municipalities with high incidence rates may also be beneficial.

Finally, additional work is needed on methods of controlling mosquito populations within septic tanks. Controlling *Ae. aegypti* productivity in septic tanks may be a method of preventing disease overwintering and reducing the burden of the disease the following rainy season. In addition to the presence of *Ae. aegypti* larvae, the septic tanks also contained *Cx. quinquefasciatus* larvae, a vector for West Nile virus. *Culex quinquefasciatus* larvae and adults were present more often and in higher numbers than *Ae. aegypti* was, and mosquito control efforts in septic tanks may help reduce West Nile transmission.

Conclusion

This study indicates that *Ae. aegypti* larvae are present in septic tanks containing raw sewage. Although this finding is inconsistent with early literature reports, it is supported by more recent findings. It is also very probable that the larvae are capable of developing from egg to adult in this environment. While it is possible that these larvae developed in a 'clean' pool of water which was outside the septic tank (e.g. a puddle in the drain pipe), several findings tend to refute this, and instead support the hypothesis that the larvae are indeed present, and developing within the septic tanks.

First, there is the fact that larvae were recovered from the tanks on multiple days and sometimes in large numbers. Due to the large surface area of the septic tanks relative to the floating funnel trap, it is unlikely that a floating funnel trap could capture multiple larvae on multiple days from a low population density. It is doubtful that an 'outside' source could produce sufficient numbers of larvae to support these trap results without being noticed by the investigators or the home owner. Second, excluding the eggs, all stages of the *Ae. aegypti* mosquito (1^{st} – 4^{th} instar larvae, pupae, and adult) were recovered from inside the floating funnel trap. It is highly unlikely that all of these stages, especially the adult, could have been recovered unless the larvae were capable of surviving, and development was occurring, inside the septic tank (and floating funnel trap) waters.

While this study was unable to determine if *Ae. aegypti* presence in septic tanks is associated with the incidence of dengue, large numbers of larvae, and especially adults, were recovered from these septic tanks. Even with significant larval mortality rates (99%), septic tanks could have a standing daily pupae count of 13.96 which equates to over 4 pupae per person. While dengue transmission is affected by herd immunity and ambient temperature, this number of pupae may be sufficient for transmission. Targeting septic tanks during the dry season, when the number of traditional surface containers is already reduced from lack of rain, may help reduce the number of pupae to levels which are too low for sustained transmission. Given *Ae. aegypti's* importance as a vector for diseases like dengue and yellow fever, mosquito programs should consider septic tanks when conducting larval surveys and developing control strategies.

Despite having a noticeably smaller funnel opening, the miniaturized Vietrap is an effective tool for sampling larval populations. Under laboratory conditions, the performance of the miniaturized Vietrap was comparable to a slightly modified model that was consistent with the size and depth of the original. The shallower draft of the miniaturized model likely offsets any reduction in performance due to the narrower funnel mouth, which is consistent with previously published findings. The narrow opening of the miniaturized Vietrap makes it especially useful for sampling subterranean structures like septic tanks which may have restricted openings.

It is also possible to develop regression plots to estimate larval population densities using the floating funnel trap. These regression plots are based on trap count results from a known larval population density. As these regression plots are generated under laboratory conditions, they may not accurately represent the true population of septic tanks. As such, they must be viewed with caution as a rough approximation of the larval population instead of a discreet value.

The use of 2,2 dichlorvos impregnated strips for controlling mosquito productivity in septic tanks does not appear to be warranted, at least not for periods longer than one month. Although the product is marketed for longer time periods, it is possible that the unique microenvironment of the septic tank led to a more rapid degradation of the strip’s active ingredient. Septic tanks are generally warm, have high levels of moisture, and contain large amounts of organic debris; all of which have been shown to either rapidly degrade pesticides, or otherwise render them inert. Although additional testing is necessary to determine the performance of other pesticides in septic tanks, the one month duration is consistent with recommended application rates; making it unlikely that another product would provide longer control.

Instead of using monthly insecticide applications to control mosquito productivity, efforts may be better directed at preventing mosquito entry into septic tanks. Although screening of vent pipes and sealing of septic tank openings and cracks may have a high initial cost, there are likely little long-term costs to such a program. Additionally, this type of intervention could be performed by the community, rather than the government. Such community based interventions are more likely to succeed in reducing the number of mosquitoes and the burden of diseases over the long-run than government insecticide programs are.

Finally, while this study was unable to develop a GIS model which could predict the incidence of dengue, this technology should be explored. Dengue surveillance in Puerto Rico is predominately reactive in nature. Samples are submitted by local physicians to the CDC laboratory in San Juan, and cases are currently reported at the municipality level.[29] Weekly case numbers are then compared to a historical average (± 2 Standard Deviations) as a means of identifying dengue outbreaks and epidemics. The problem with this method is that while a rapid response may decrease the magnitude of the outbreak, an outbreak has still occurred.

In order to minimize an outbreak response time, effective surveillance systems must not only track disease incidence, but they should also be able to predict outbreaks before they occur. Geographic information systems are another tool which may be useful to not only explain past dengue outbreaks in Puerto Rico, but also predict new outbreaks before they occur. Several environmental and socioeconomic variables in this study were associated with dengue incidence. While none of the variables, either alone or in conjunction with the others, adequately explained the incidence of dengue in southeast Puerto Rico, the associations were significant. Given the fact that GIS models for predicting dengue have been successfully developed in other countries, further efforts should be made to develop one in Puerto Rico as well. Using smaller geographic areas of interest, especially concerning weather data, may help with the development of a suitable model. Finally, the electronic reporting of patient addresses will enable this model to be extended to the entire island, and not limited to just the southeast region.

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Appendices

Appendix 1 – Water and Septic Tank Usage

1. Do you use a septic tank? **Y N**

2. How many people live in your house? **1 2 3 4 5 More than 5**

3. Does your family usually take a shower or bath? **Shower Bath**

4. On average, how many showers (baths) does each person take per day? **1 2 3 or more**

5. How long is the average shower**?**

Less than 5 minutes 5-10 minutes More than 10 minutes

6. Do you use a dishwashing machine? **Y N**

7. If yes, how often do you use it? **Weekly 2-3 times/week Daily**

8. Do you have a washing machine for clothes? **Y N**

9. If yes, how often do you use it?

Weekly 2-3 times/week Once/day More than once/day

10. Where does the septic tank water come from? (circle all that apply)

Toilet Shower/Bath Kitchen Laundry Don't Know

11. Where is your water from?

Private well Public (city) well Don't know

12. How often is your septic tank pumped out?

More than once/month Once a month 3-4 times/year

2 times per year Once a year Less than once a year Don't know

13. How large is your septic tank (in gallons)?

Less than 2,000 2,000 3,000 4,000 5,000

More than 5,000 Don’t know

14. What is your tank constructed of?

Concrete only Concrete and Steel Plastic Other Don’t Know

15. How old is your septic tank?

Less than 5 years 5-10 years 10-15 years 15-20 years More than 20 years

Don’t know

Appendix 2 – Field Observations

Environmental conditions

Distance from house (in meters)

Opening covered/sealed

Opening distance from walls

Vent pipe screened

Vent pipe length

Cracks in tank cover

Direct sun exposure

Above ground height

Tank dimensions

Water Quality

Day 1	pH		Temperature		TDS	
Day 2	pH		Temperature		TDS	
Day 3	pH		Temperature		TDS	
Day 4	pH		Temperature		TDS	

Adult presence

	Aedes aegypti	*Culex quinquefasciatus*
Day 1		
Day 2		
Day 7		

Larval presence

	Aedes aegypti	*Culex quinquefasciatus*
Day 1		
Day 2		
Day 3		

Day 4		