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Patterns and Mechanisms of the Geographic Expansion of *Aedes aegypti* in the Peruvian Amazon

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Population Biology, Ecology, and Evolution

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Sarah Anne J. Guagliardo

B.S., The George Washington University, 2005
M.P.H., Yale University, 2010

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Abstract

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Sarah Anne J. Guagliardo

Transmitted to humans through the bites of infected *Aedes* mosquitoes, dengue is the world's most important arbovirus, with nearly 2.5 billion people at risk for disease. Although present in the Americas for centuries, the primary dengue vector *Aedes aegypti* is expanding from urban to peri-urban and rural areas throughout Latin America after its near continental elimination in the 1950's. In the Peruvian Amazon, *Ae*. *aegypti* is abundant in urban centers such as Iquitos (pop: ~400,000), and in recent years, has also been found in a number of neighboring communities. *Ae. aegypti* active dispersal (through flight) is limited to <100m, and therefore long-distance dispersal must be facilitated through human activity. In this dissertation I: 1) assess risk factors for *Ae. aegypti* invasion through entomological surveys in 34 communities; 2) clarify the relative importance of different vehicles (i.e.- boats, trucks) in the invasion process; and link vehicle infestation with transportation data to measure the frequency and intensity of new introductions ("propagule pressure"); 3) measure oviposition frequency on river boats; and 4) characterize gene flow using 10 microsatellite markers and linked such data to long-distance dispersal mechanisms. Taken together, evidence from this dissertation highlights the importance of river boats as major drivers of *Ae. aegypti* regional expansion. My main findings are: 1) risk for invasion is a function of connectivity and proximity to major urban centers; 2) several vehicle types are responsible for transporting immature and adult mosquitoes (mostly barges); 3) few individual barges produce the majority of mosquitoes, acting as "super-transporters." These results have important implications for dengue control: many novel control strategies (i.e.- genetically modified "sterile" mosquitoes) falsely assume that mosquito populations are immobile, whereas my results show continual gene flow among *Ae. aegypti* metapopulations. From an invasion ecology perspective, I show that propagule pressure can be more precisely quantified through field surveys. I also propose a new method for evaluating genetic isolation by distance through the "Propagule Pressure Index," combining transportation data with vehicle infestation rates. Broadly, results from this study can help anticipate vector population mixing and future range expansions of dengue and other viruses transmitted by *Ae. aegypti*.

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Chapter 1: Introduction

1.1 Emerging and Re-emerging Infectious Diseases

It is estimated that more than 25% of deaths worldwide are attributable to infectious causes, not including deaths from past infections or chronic infections [1]. The burden of infectious disease falls disproportionately on people living in developing countries, with children and marginalized populations at greatest risk [2]. In the context of advanced economies, minority and disenfranchised groups such as indigenous people suffer disproportionately more from infectious diseases [3]. Despite significant advances in medicine and public health in the 20th century (i.e. - vaccine development, improved surveillance, antimicrobials, mosquito control tools), much of the world continues to suffer from infectious causes, and new challenges are presented by the development of antimicrobial resistance and global social and demographic changes.

Emerging infectious diseases (EIDs) can be defined as “infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range” [4]. The burden of disease due to emerging and re-emerging pathogens varies greatly from severe epidemics (such as emergence of Acquired Immune Deficiency Syndrome) to relatively minor but strange events (such as a 2003 monkey pox outbreak in North America [5]). Still, EIDs have resulted in serious consequences throughout the course of human history; the Black Death of 14th century Europe is estimated to have resulted in 50 million deaths [6], European-introduced smallpox and measles viruses decimated Pacific and American indigenous populations [7-9], and the influenza pandemic of 1918 resulted in more fatalities than World War I [10].

The global emergence of novel pathogens and re-emergence of existing pathogens is thought to be driven by a combination of ecological, socioeconomic and environmental factors [1,11-18]. Biological factors such as the evolution of antimicrobial resistance are important for EIDs, but social and environmental determinants can be just as relevant. Indeed, the social factors that led to the emergence of AIDS (warfare, natural disasters, poverty, environmental disturbance, and the degradation of social connections) are similar to those that led to the emergence of other important epidemics such as diphtheria, plague, and cholera [1,19-21].

Wildlife zoonotic and vector-borne EIDs pose a particular threat in tropical areas, where surveillance and reporting efforts are lagging [11]. In other words, regions of the world from which EIDs are most likely to originate are least likely to actively monitor and report the emergence of novel pathogens. For this reason, a comprehensive understanding of the social, biological, and environmental dimensions of disease emergence in developing countries is paramount for prevention and mitigation efforts.

1.2 Dengue Fever Biology, Ecology, and Epidemiology

Dengue Virus Biology

Dengue fever is a severe acute viral disease cause by infection with dengue virus (DENV), and is transmitted to humans through the bites of infected mosquitoes. DENV is an enveloped, single stranded RNA positive-strand virus of the family *Flaviviridae*, genus *Flavivirus* [22,23], and is composed of 11,000 nucleotide bases that code for three structural proteins, seven nonstructural proteins, and non-coding regions [24,25].

There are four antigenically distinct DENV serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, classified on the basis of their surface antigens. Upon infection, life-long immunity is conferred against the serotype of infection, while temporary immunity is conferred across serotypes. Subsequent infection with a different serotype results elevated viremia and risk for severe disease [26-30], which may include outcomes such as hemorrhagic fever and death. The proposed mechanism for this phenomenon is known as Antibody Dependent Enhancement (ADE), in which sub-neutralizing antibodies from the primary infection bind to the new virus serotype in secondary infections. Virus entry into myeloid cells (monocytes and macrophages) then enables endocytosis through the Fcγ receptor [31]. The virus can then escape the phagolysosome and utilize the host cell to produce new virions [32]. Several dengue trial vaccines were underway at the time of writing this dissertation and more have been underway for decades, so far with limited results [33-39]. ADE presents serious challenges - a monovalent vaccine might actually result in disease outcomes far more serious than primary infection. Of course, vaccines are also limited by challenges associated with financing, supply, and administration and delivery.

Dengue Virus Epidemiology

DENV was maintained in a sylvatic cycle between non-human primates and *Aedes* genus mosquitoes in Africa and Asia, and it is thought that several distinct spillover events led to the emergence of the four viral serotypes approximately 500 to 1000 years ago [40]. Today, urban DENV is maintained in a non-zoonotic transmission cycle between humans and *Aedes* genus mosquitoes, with *Aedes aegypti* being the most common vector worldwide.

Dengue fever is widely regarded as the world's most important arboviral disease, with nearly 2.5 billion people at risk for disease worldwide, as estimated by the World Health Organization [41]. Dengue epidemiology varies spatially and temporally, with severe underreporting in some regions of the world. A recent study by Bhatt et al (2013) estimated the total dengue infections per year to be 390 million, 96 million of which are apparent infections (with clear symptoms of disease) [42]. The actual number of reported dengue cases is approximately 70 to 100 million every year, with approximately 2.1 million cases of life-threatening disease in the form of Dengue Hemorrhagic Fever (DHF)/Dengue Shock Syndrome (DSS) [43].

Over the last two decades, the burden of disease due to dengue fever has greatly increased, with endemic (continued incidence) and hyperendemic (continued, high incidence) transmission of multiple serotypes becoming more common [44]. Human population growth, rapid and unplanned urbanization, increasing inequalities, and human travel have contributed to the resurgence and spread of DENV infections [45]. The most dramatic changes in DENV epidemiology have occurred in the Americas: the resurgence of the disease has coincided with the resurgence of *Ae. aegypti* mosquito populations [46]. In the mid-twentieth century (1946-1963), *Ae. aegypti* populations were dramatically reduced following a yellow-fever control program led by the Pan American Health Organization [47,48]. The successful reduction of yellow fever (also spread by *Ae. aegypti*) led to the waning of control programs targeting the mosquito, and as a result, *Ae. aegypti* was reestablished in countries throughout the Americas since the 1960s [49,50]. This resulted in the re-emergence of dengue: since the 1980s, dengue epidemics have been regularly occurring in American countries that had been dengue-free for 35 or more years, and by 1994, all four serotypes were present in the Americas [46].

In the absence of a vaccine or cure for dengue, control programs rely on the suppression of *Ae. aegypti* populations to limit virus transmission. Accordingly, understanding the biology, ecology, behavior, and distribution of this vector remains essential for disease control.

1.3 *Aedes aegypti* Biology, Ecology, and Global Expansion

The spatial distribution of DENV infections is primarily constrained by the distribution of the daytime-biting *Ae. aegypti* [51]. All mosquitoes have four life stages; eggs, larvae, pupae, and adults. *Ae. aegypti* eggs exhibit extreme resistance to desiccation, surviving up to two years (and possibly longer) without water or nutrients [52,53]. Females lay their eggs on the edges of water-holding artificial containers, and at the larval stages, *Ae. aegypti* feed on microorganisms and other organic matter that accumulate in these containers over time. Larvae shed their skin three times before reaching the fourth instar stage, after which pupation occurs. *Ae. aegypti* pupae do not feed but undergo physical changes that lead to the emergence of adult mosquitoes. The entire life cycle typically lasts approximately two to four weeks, but survival is highly dependent on environmental conditions, mainly temperature and the availability of nutrients (organic matter in water at the larval stage or blood meals at the adult stage) [54].

Ae. aegypti mosquitoes are well-adapted to the human environment: females feed almost exclusively from humans, and prefer to rest in dark, cool areas, usually indoors [45]. Adult females lay their eggs on the walls of water-filled artificial containers found in and around the home such as vases, plastic buckets, water storage tanks, and discarded refuse and tires [54]. This extraordinary adaption to human environments, in conjunction with egg resistance to desiccation, has contributed to the global spread and establishment of this mosquito vector.

Though originally African in origin, *Ae. aegypti* has been present in the Americas for centuries. It was most likely accidentally transported via European ships used for slave trade, and through the transport of goods, colonization, and exploration during 17th–19th centuries [55]. DENV epidemics were common in ports cities as urbanization continued and the shipping industry expanded in the 18th-20th centuries [56]. By the twentieth century, *Ae. aegypti* was present throughout North and South Americas, probably first infesting port cities and then moving inland [55,56]. As previously mentioned, the waning of an effective PAHO-sponsored yellow fever control program has resulted in the geographic expansion of *Ae. aegypti* (and DENV) in the Americas. Since the 1970s, *Ae. aegypti* populations have been re-invading urban areas, and newly invading semi-urban and rural areas. Despite this apparent range expansion, the invasion of *Ae. aegypti* has largely been overshadowed by the global expansion of the related Asian tiger mosquito *Aedes albopictus*. As the primary vector of DENV, it is of utmost importance to understand the spatial distribution and population dynamics of *Ae. aegypti* mosquitoes. In this dissertation I use an invasion ecology lens to further scientific understanding of *Ae. aegypti* expansion.

1.4 Theoretical Foundations of Invasion Ecology

Invasion ecologists think of the invasion process in a sequence of steps including; 1) uptake from the native range, 2) transport to a new region (often facilitated by human transport), 3) release into the new environment, and 3) establishment and spread in the new environment (**Figure 1.1**) [57]. Successful invasion requires completion of all three sequential steps. Invasion success is determined by several factors including the number and frequency of introduction events (propagule pressure) [58,59], behavior of the invader [60], abiotic and biotic properties of the receiving ecosystem [61-64], or their combined effect [65,66]. Understanding the mechanisms underlying these invasion steps is critical for preventing, reducing, and predicting the future expansion of any invader, and in the case of invasive vectors, the disease it causes.

In the invasion ecology literature, the propagule pressure has received much attention as an explanation of invader success. Propagule pressure is defined as “a composite measure of the number of individuals released into a region to which they are not native” [57,67]. Propagule pressure takes into consideration both the raw number of individuals released during an introduction event (propagule size) and the frequency of introduction events (propagule number) [57]. There are several ways in which movement between locations may influence propagule pressure, and therefore, probability of successful invasion. For example, transport vehicle type (i.e.- boat vs. truck) is likely to be a determinant of propagule size, as different vehicles may exhibit environments that are more or less hostile to invaders. In addition, the transport vehicle’s frequency of travel is an important determinant of propagule number.

Invaders arrive in new locations in one of numerous ways; through a human transport vehicle (i.e.- boats, cars), through the arrival of goods, and/ or natural dispersal such as wind or ocean currents [68]. Indeed, successful biological invasion is an outcome that is a result of several interacting processes, and at each stage of the process, the probability of success decreases.

1.5 Dissertation Objectives

Broadly, the research compelling this dissertation seeks to characterize the patterns of *Ae. aegypti* expansion in the Peruvian Amazon and identify the underlying mechanisms that drive this expansion. From a theoretical perspective, I expand upon previous work in *Ae. aegypti* invasion by directly linking human transportation networks to the spread of invasive species. I describe these theoretical contributions below, in order of chapter.

In **Chapter 2** of this dissertation I address the question: What factors are associated with successful *Ae. aegypti* establishment at the community, household, and container scales? I thoroughly characterize the spatial distribution of *Ae. aegypti* mosquitoes at these ecological scales and link mosquito infestation with risk factors at each scale. To my knowledge, such an approach for invasive mosquitoes has not yet been employed. Of particular interest is the characterization of mosquito infestation at the community scale: although many studies have conducted entomological collections at the household and container-levels, only two have utilized this technique for towns or communities [69,70]. Results from this study showed that geographic distance from major population centers and human population size are predictive of *Ae. aegypti* presence at the community scale, indicating that gravity models may be an appropriate way to model *Ae. aegypti* spread.

In **Chapter 3**, I explore human vehicle (cars, boats, etc) infestation rates as a potential explanatory variable for *Ae. aegypti* spread across long distances. In the past, invasion ecologists have pointed to human transport vehicles as important vessels for the spread of invasive species. To my knowledge, however, no study has ever addressed the relative contribution of different vehicle types to the spread of invasive species over different seasons. In the context of invasive mosquitoes, Morrison et al (2006) and Bataille et al (2009) characterized infestation on boats and planes, respectively, using entomological surveys

[71,72]. I build upon their approach by comparing large barges with other vehicle types (i.e. – buses) and thoroughly characterizing infestation by floor and container types. I show that some vehicle types are much more likely than others to contain *Ae. aegypti*, and that a small proportion of boats produce the vast majority of mosquitoes.

In **Chapter 4**, I report the results of the placement of baited ovitraps on large barges to monitor *Ae. aegypti* oviposition throughout the course of a year. To my knowledge, no study has previously used traps to actively monitor invasive organisms on human vehicles. Ovitraps have the potential to be used as a tool to both monitor and control mosquito populations on boats.

Lastly, in **Chapter 5**, I use population genetics to demonstrate that *Ae. aegypti* expansion in the Peruvian Amazon is driven by boater traffic. I take the unique approach of directly linking transportation network data (collected through interviews) with genetic distance data (F_{ST}) to show that mosquito range expansion is ultimately correlated with river traffic patterns in the Amazon. Although previous research in the population genetics literature has inferred that human transportation networks are relevant for *Ae. aegypti* spread [73-75] , to date none have thoroughly explored the mechanisms driving this phenomenon.

1.6 Study Area

The Amazonian city of Iquitos, Department of Loreto, Peru represents a unique setting in which to study these questions. Nearly 630 miles from Lima, Iquitos is the largest city in the Peruvian Amazon, resting at the intersection of the Itaya, Nanay, and Amazon Rivers. The city itself has approximately 400,000 inhabitants, with a number of smaller settlements along a highway running from Iquitos to the much smaller town, Nauta (pop: 16,000). In addition, there are a number of settlements along the rivers outside of Iquitos. Accordingly, the two modes of transport in the region are via rivers and via the Iquitos-Nauta highway. Some of these communities have access to both river and road transport, while the majority are only accessible by one or the other.

Ae. aegypti was first reported in Peru in 1852, and was declared eradicated in 1958 following the success of the PAHO yellow fever control program [76,77]. In 1984, *Ae. aegypti* reappeared in Iquitos, later also appearing in other cities throughout Loreto and the rest of Peru [78]. DENV was reintroduced in Peru 1990, with the first modern documented case occurring in Iquitos, [79]. Today, all four dengue serotypes circulate in Iquitos: DENV-1 invaded in 1990-91, an American strain of DENV-2 in 1995 [80,81], DENV-3 in 2001 [82], an Asian variant of DENV-2 in 2002, and DENV-44 in 2008 [83].

1.7 Figures

Figure 1.1. Steps in the invasion process. The invasion process can be organized into a sequence of steps including; existence in the native range, uptake from the native range, transport (usually facilitated by a human vehicle), release into the new environment, and establishment and spread.

1.8 References Cited

1. Morens DM, Folkers GK, Fauci AS (2004) The challenge of emerging and re-emerging infectious diseases. Nature 430: 242-249.

2. Guerrant RL, Blackwood BL (1999) Threats to global health and survival: the growing crises of tropical infectious diseases--our "unfinished agenda". Clin Infect Dis 28: 966-986.

3. Butler JC, Crengle S, Cheek JE, Leach AJ, Lennon D, et al. (2001) Emerging infectious diseases among indigenous peoples. Emerg Infect Dis 7: 554-555.

4. Morse SS (1995) Factors in the emergence of infectious diseases. Emerg Infect Dis 1: 7-15.

5. Centers for Disease Control Prevention (2003) Multistate outbreak of monkeypox--Illinois, Indiana, and Wisconsin, 2003. MMWR Morb Mortal Wkly Rep 52: 537-540.

6. Kohn GC (1995) Facts on File. In: Kohn GC, editor. The Wordsworth Encyclopedia of Plague and Pestilence. New York.

7. Hopkins D (1983) Smallpox in History Princes and Peasants. Chicago: Univ. Chicago Press. pp. 204–233.

8. MacArthur N (1968) Island Populations of the Pacific Canberra: Australian National Univ. Press.

9. De Bevoise K (1995) Agents of Apocalypse: Epidemic Disease in the Colonial Philippines. Princeton: Princeton Univ. Press.

10. Johnson NP, Mueller J (2002) Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med 76: 105-115.

11. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, et al. (2008) Global trends in emerging infectious diseases. Nature 451: 990-993.

12. Smolinski MS, Hamburg MA, Lederberg J, editors. Microbial Threats to Health: Emergence, Detection, and Response. Washington, D.C.: The National Academies Press; 2003.

13. Binder S, Levitt AM, Sacks JJ, Hughes JM (1999) Emerging infectious diseases: public health issues for the 21st century. Science 284: 1311-1313.

14. Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287: 443-449.

15. Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci 356: 983-989.

16. Patz JA, Daszak P, Tabor GM, Aguirre AA, Pearl M, et al. (2004) Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence. Environ Health Perspect 112: 1092-1098.

17. Weiss RA, McMichael AJ (2004) Social and environmental risk factors in the emergence of infectious diseases. Nat Med 10: S70-76.

18. Woolhouse ME, Gowtage-Sequeria S (2005) Host range and emerging and reemerging pathogens. Emerg Infect Dis 11: 1842-1847.

19. Desselberger U (2000) Emerging and re-emerging infectious diseases. J Infect 40: 3-15.

20. Pollard AJ, Dobson SR (2000) Emerging infectious diseases in the 21st century. Curr Opin Infect Dis 13: 265-275.

21. Feldmann H, Czub M, Jones S, Dick D, Garbutt M, et al. (2002) Emerging and re-emerging infectious diseases. Med Microbiol Immunol 191: 63-74.

22. Lindenbach BD, Rice CM (2003) Molecular biology of flaviviruses. Adv Virus Res 59: 23-61.

23. Harris E, Holden KL, Edgil D, Polacek C, Clyde K (2006) Molecular biology of flaviviruses. Novartis Found Symp 277: 23-39; discussion 40, 71-23, 251-253.

24. Rodenhuis-Zybert IA, Wilschut J, Smit JM (2010) Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci 67: 2773-2786.

25. Hanley KA, Weaver SC. Frontiers in Dengue Virus Research. Caister Academic Press; 2010.

26. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 181: 2-9.

27. Halstead SB (2003) Neutralization and antibody-dependent enhancement of dengue viruses. Adv Virus Res 60: 421-467.

28. Halstead SB, Simasthien P (1970) Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. Yale J Biol Med 42: 276-292.

29. Chareonsirisuthigul T, Kalayanarooj S, Ubol S (2007) Dengue virus (DENV) antibody-dependent enhancement of infection upregulates the production of anti-inflammatory cytokines, but suppresses anti-DENV free radical and pro-inflammatory cytokine production, in THP-1 cells. J Gen Virol 88: 365-375.

30. Kou Z, Lim JY, Beltramello M, Quinn M, Chen H, et al. (2011) Human antibodies against dengue enhance dengue viral infectivity without suppressing type I interferon secretion in primary human monocytes. Virology 410: 240-247.

31. de Alwis R, Williams KL, Schmid MA, Lai CY, Patel B, et al. (2014) Dengue viruses are enhanced by distinct populations of serotype cross-reactive antibodies in human immune sera. PLoS Pathog 10: e1004386.

32. Rodenhuis-Zybert IA, van der Schaar HM, da Silva Voorham JM, van der Ende-Metselaar H, Lei HY, et al. (2010) Immature dengue virus: a veiled pathogen? PLoS Pathog 6: e1000718.

33. Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet 380: 1559-1567.
34. Guy B, Barrere B, Malinowski C, Saville M, Teyssou R, et al. (2011) From research to phase III: preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. Vaccine 29: 7229-7241.
35. Osorio JE, Huang CY, Kinney RM, Stinchcomb DT (2011) Development of DENVax: a chimeric dengue-2 PDK-53-based tetravalent vaccine for protection against dengue fever. Vaccine 29: 7251-7260.
36. Durbin AP, Kirkpatrick BD, Pierce KK, Elwood D, Larsson CJ, et al. (2013) A single dose of any of four different live attenuated tetravalent dengue vaccines is safe and immunogenic in flavivirus-naive adults: a randomized, double-blind clinical trial. J Infect Dis 207: 957-965.
37. Durbin AP, Kirkpatrick BD, Pierce KK, Schmidt AC, Whitehead SS (2011) Development and clinical evaluation of multiple investigational monovalent DENV vaccines to identify components for inclusion in a live attenuated tetravalent DENV vaccine. Vaccine 29: 7242-7250.
38. Coller BA, Clements DE, Bett AJ, Sagar SL, Ter Meulen JH (2011) The development of recombinant subunit envelope-based vaccines to protect against dengue virus induced disease. Vaccine 29: 7267-7275.
39. Danko JR, Beckett CG, Porter KR (2011) Development of dengue DNA vaccines. Vaccine 29: 7261-7266.
40. Wang E, Ni H, Xu R, Barrett AD, Watowich SJ, et al. (2000) Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. J Virol 74: 3227-3234.

41. World Health Organization [Internet]. Geneva: Dengue and severe dengue; 2014 [updated February 2015]. Available from: http://www.who.int/mediacentre/factsheets/fs117/en/

42. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. Nature 496: 504-507.

43. Kyle JL, Harris E (2008) Global spread and persistence of dengue. Annu Rev Microbiol 62: 71-92.

44. Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10: 100-103.

45. Halstead SB (2008) Dengue virus-mosquito interactions. . Annu Rev Entomol 53: 273-291.

46. Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-496.

47. Soper FL, Wilson DB, Lima S, Sá Antunes W, Rockefeller Foundation. The organization of permanent nation-wide anti-Aedes aegypti measures in Brazil. New York,: The Rockefeller Foundation; 1943.

48. Schliessmann DJ, Calheiros LB (1974) A review of the status of yellow fever and *Aedes aegypti* eradication programs in the Americas. Mosq News 34: 1-9.

49. Bres P (1979) Historical review of dengue 1: Implication of its introduction in the western hemisphere in 1977. In: Caribbean WGoDit, editor. Washington, D.C.: Pan American Health Organization. pp. 4-10.

50. Gubler DJ (1989) *Aedes aegypti* and *Aedes aegypti*-borne disease control in the 1990s: top down or bottom up. Am J Trop Med Hyg 40: 571-578.

51. Reiter P, Gubler DJ (1997) Surveillance and control of urban dengue vectors. In: Gubler DJ, Kuno G, editors. Dengue and dengue hemorragic fever. Wallingford, UK CAB International.

52. Sota T, Mogi M (1992) Interspecific variation in desiccation survival time of *Aedes* (Stegomyia) mosquito eggs is correlated with habitat and egg size. Oecologia 90: 353-358.

53. Juliano SA, Lounibos LP (2005) Ecology of invasive mosquitoes: effects on resident species and on human health. Ecol Lett 8: 558-574.

54. Mosquitoes' main aquatic habitats. Dengue Entomology and Ecology: Centers for Disease Control and Prevention.

55. Nelson MJ (1986) Aedes aegypti: Biology and Ecology; Organization PAH, editor. Washington, D.C.

56. Gubler DJ, Kuno G (1997) Dengue and dengue hemorrhagic fever. Wallingford, Oxon, UK ; New York: CAB International. xi, 478 p. p.

57. Lockwood JL, Cassey P, Blackburn T (2005) The role of propagule pressure in explaining species invasions. Trends Ecol Evol 20: 223–228.

58. Lockwood JL, Cassey P, Blackburn TM (2009) The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. Divers Distrib 15: 904-910.

59. Williamson MH (1996) Biological invasions. London; New York: Chapman & Hall.

60. Chapple DG, Simmonds SM, Wong BBM (2012) Can behavioral and personality traits influence the success of unintentional species introductions? Trends Ecol Evol 27: 57-64.

61. Burke MJW, Grime JP (1996) An Experimental Study of Plant Community Invasibility. Ecology 77: 776-790.

62. Moyle PB, Light T (1996) Biological invasions of fresh water: Empirical rules and assembly theory. Biol Cons 78: 149-161.

63. Holway DA (1998) Factors governing rate of invasion: a natural experiment using Argentine ants. Oecologia 115: 206-212.

64. Levine JM, D'Antonio CM (1999) Elton revisited: a review of evidence linking diversity and invasibility Oikos 87: 15-26.

65. Williamson J HS (2002) Biotic and abiotic limits to the spread of exotic revegetation species. Ecological Applications 12: 40-51.

66. Lonsdale WM (1999) Global patterns of plant invasions and the concept of invasibility. Ecology 80: 1522-1536.

67. Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. J Appl Ecol 46: 10–18.

68. Carlton JT (1996) Pattern, process, and prediction in marine invasion ecology. Biological Conservation 78: 97-106.

69. Diaz-Nieto LM, Macia A, Perotti MA, Beron CM (2013) Geographical limits of the Southeastern distribution of *Aedes aegypti* (Diptera, Culicidae) in Argentina. PLoS Negl Trop Dis 7: e1963.

70. Troyes L, Villegas Z, Troyes M (2006) Expansión del *Aedes aegypti* a localidades rurales de Cajamarca Rev Perú Med Exp Salud Pública 23: 163-167.

71. Morrison AC, Sihuincha M, Stancil JD, Zamora E, Astete H, et al. (2006) Aedes aegypti (Diptera: Culicidae) production from non-residential sites in the Amazonian city of Iquitos, Peru. Ann Trop Med Parasitol 100 Suppl 1: S73-S86.

72. Bataille A, Cunningham AA, Cedeno V, Cruz M, Eastwood G, et al. (2009) Evidence for regular ongoing introductions of mosquito disease vectors into the Galapagos Islands. Proc Biol Sci 276: 3769-3775.

73. de Lourdes Munoz M, Mercado-Curiel RF, Diaz-Badillo A, Perez Ramirez G, Black WCt (2013) Gene flow pattern among Aedes aegypti populations in Mexico. J Am Mosq Control Assoc 29: 1-18.

74. Huber K LL, Chantha N, Failloux AB (2004) Human transportation influences Aedes aegypti gene flow in Southeast Asia. Acta Tropica 90: 23-29.

75. da Silva AG CI, Santos WS, Luz LB, Ribolla PEM, Abad-Franch F (2012) Gene flow networks among American Aedes aegypti populations. Evolutionary Applications 5: 664-676.

76. Andrade CS, Caceres AG, Vaquerizo A, Ibanez-Bernal S, Cachay LS (2001) Reappearance of Aedes aegypti (Diptera: Culicidae) in Lima, Peru. Mem Inst Oswaldo Cruz 96: 657-658.

77. Griffitts DHT (1934) Fiebre amarilla: importancia de algunas fases relativamente nuevas en relación con su profilaxia en los focos antiguos y nuevos. . Bol Of Sanit Panam 13: 511-518.

78. Phillips I, Need J, Escamilla J, Colan E, Sanchez S, et al. (1992) First documented outbreak of dengue in the Peruvian Amazon region. Bull Pan Am Health Organ 26: 201-207.

79. Phillips I, Need J, Escamilla J, Colan E, Sánchez S, et al. (1992) First documented outbreak of dengue in the Perúvian Amazon Region. Bull Pan Am Health Organ 26: 201-207.

80. Watts DM, Porter KR, Putvatana P, Vasquez B, Calampa C, et al. (1999) Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. Lancet 354: 1431-1434.

81. Hayes CG, Phillips IA, Callahan JD, Griebenow WF, Hyams KC, et al. (1996) The epidemiology of dengue virus infection among urban, jungle, and rural populations in the Amazon region of Peru. Am J Trop Med Hyg 55: 459-463.

82. Kochel T, Aguilar P, Felices V, Comach G, Cruz C, et al. (2008) Molecular epidemiology of dengue virus type 3 in Northern South America: 2000--2005. Infect Genet Evol 8: 682-688.

83. Forshey BM, Morrison AC, Cruz C, Rocha C, Vilcarromero S, et al. (2009) Dengue virus serotype 4, northeastern Peru, 2008. Emerg Infect Dis 15: 1815-1818.

Chapter 2: Patterns of Geographic Expansion of *Aedes aegypti* in the Peruvian Amazon

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2.1 Introduction

Aedes aegypti is the vector of several arboviruses of major global health importance, including dengue virus (DENV), yellow fever virus, and chikungunya and Mayaro viruses. Of these, dengue virus is the most prevalent and geographically extensive, with approximately 2.5 billion people at risk worldwide [1], and 390 million new dengue infections each year [2]. This mosquito vector is well-adapted to the urban environment: females feed almost exclusively on humans, prefer to rest in dark, cool areas (usually indoors) [3], and adult female mosquitoes lay their eggs on the walls of water-filled artificial containers found in and around the home such as vases, plastic buckets, water storage tanks, and discarded refuse and tires [4]. These adaptations to human environments, coupled with the longevity and resistance of its eggs to desiccation [5, 6], contribute to the vector's passive spread to new areas via human transportation networks [7]. In the absence of a vaccine or cure for dengue, most dengue control programs rely on the suppression of vector populations to prevent human exposure to infected mosquitoes. Accordingly, understanding the geographic distribution and range expansion of this vector is of utmost importance for disease surveillance and control.

Originally African in origin, it is thought that *Ae. aegypti* was transported inadvertently to the Americas via European ships used for trade, commerce, and slave transport in the 17th–19th centuries [8]. As urbanization continued and the shipping industry expanded, outbreaks of dengue-like illnesses became more common in port cities [8]. By the 20th century, *Ae. aegypti* was present throughout North and South America, probably first infesting port cities and then moving inland [8, 9]. During the mid-20th century (1946-1963), *Ae. aegypti* populations in the Americas were dramatically reduced as a result of a yellow fever control program led by the Pan American Health Organization [10, 11]. The successful reduction of yellow fever led to the waning of control programs targeting the mosquito, and consequentially, since the 1980s, *Ae. aegypti* has become reestablished throughout the Americas [12].

In Latin America and in Peru, *Ae. aegypti* mosquitoes are in the process of expanding from urban to rural areas [13, 14]. The Amazonian city of Iquitos was the first site of *Ae. aegypti* (in 1984) and dengue (in 1990) reestablishment in Peru [15]. Iquitos rests at the intersection of the Amazon, Nanay, and Itaya Rivers which connect it to a number of smaller settlements throughout the region. Despite apparently similar climatic and socioeconomic conditions shared by most communities in the region, *Ae. aegypti* is heterogeneously distributed among these rural settlements.

Invasion ecologists describe the invasion process as a series of sequential steps that include transport to a new area, release, establishment, and spread [16]. Invasion success is determined by several factors including the number and frequency of introduction events (propagule pressure) [17, 18], abiotic and biotic properties of the receiving ecosystem [19–22], and behavior of the invader (e.g., tolerance of or attraction to human environments, oviposition behavior) [16]. In this study, we focus on introductory pressure (by measuring the number of vehicles and trips traveling to and from each town), selected abiotic factors (e.g., container abundance), and biotic factors (e.g., presence of other mosquito species). Understanding the mechanisms underlying these invasion steps for *Ae. aegypti* is critical for predicting and mitigating future expansion of this mosquito species and the pathogens it transmits.

The region surrounding Iquitos provides an ideal setting to study these questions, due to the presence of an interconnected network of settlements with different population sizes, varying degrees of urbanization, reliance on both river and road transit, and similar climatic conditions (e.g., temperature, rainfall) at this ecological scale. In this context, we used two pupal-demographic datasets (an existing historical dataset from 2008-11 and more in-depth data collected for this study from 2011-12) to address two questions: 1) Which factors are associated with *Ae. aegypti* establishment in rural areas?; and 2) How do different human transportation networks influence *Ae. aegypti* spread (rivers vs. roads, primary vs. secondary transportation routes)?

2.2 Methods

Ethics Statement

No personal information was collected during interviews. Permission for this study was granted by the Loreto Regional Health Department, and the study protocol was approved by the NAMRU-6 Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects (protocol number NAMRU6.2012.0039). In addition, the Emory University Institutional Review Board determined that this study does not represent human subjects based research.

Study Area

With approximately 380,000 inhabitants, Iquitos is the largest population center in the Department of Loreto, Peru. Transportation pathways, including new roads and river routes have been developed over the course of the past 30 years as a result of increased commerce and trade in natural resources (e.g., oil, timber, and coca). As a result, small settlements in the region have experienced rapid population growth and expansion [23]. Although river networks are the predominant mode of transit, a 95 km road connecting Iquitos to the smaller city of Nauta (population: 17,000) facilitates terrestrial commerce and population movement. With its construction, the road has brought about the establishment of new settlements in areas that were previously inaccessible, human population growth (growth rate approximately 4% greater than that of Iquitos), and deforestation due to farming [24]. Communities included in the present study

(**Table S2.1**) can largely be described as “rural” due to small population sizes (ranging from ~100 to 6,000 inhabitants), geographic isolation from large cities, and limited access to cellular networks and other communication channels. People living in these communities subsist on hunting, fishing, and small-scale agriculture, such as cassava root and plantain farming [25]. Water is derived from a variety of sources: piped water systems (accessible in the home via a faucet but not potable), water collected in buckets directly from the river, rain water that is actively gathered in large drums from roofs either directly or from gutters, and well water from ground sources. Unmanaged or discarded containers may also be passively filled through the unintentional accumulation of rain water. A map of the study area is shown in **Figure 2.1**.

Datasets

We used historical data available from the Peruvian Ministry of Health (MOH) and Naval Medical Research Unit No. 6 (NAMRU-6) to characterize patterns of *Ae. aegypti* expansion at the community scale. NAMRU-6 personnel conducted *Ae. aegypti* pupal-demographic surveys as part of epidemiological studies on alphaviruses, while the Peruvian MOH independently carried out larval surveys as a component of normal surveillance activities. The MOH and NAMRU-6 data consisted of information about 31 communities and two cities in the region during 2008, 2011, and 2012, and were obtained by surveying approximately 10% of houses in a community [26]. Each house was searched thoroughly for *Ae. aegypti* mosquito larvae and pupae. In addition, in NAMRU-6 surveys Prokopack aspirators were used to collect adult mosquitoes [27].

To supplement these historical data we selected communities for a more detailed analysis of*Ae. aegypti* presence. To determine mosquito presence in 2011-12, we deployed ovitraps and simultaneously conducted a thorough survey of wet containers (with water at the time they were surveyed) within households (described in further detail below). Communities were selected along two transects: one following the Iquitos- Nauta highway (N = 22) and the other along the Amazon River (N = 12), for a total of 34 communities. The geographic limit of transects was defined by the network distance (travel time) from Iquitos, under the assumption that long-distance dispersal of *Ae. aegypti* is due to unintentional passive human transport of immature and adult mosquitoes. For the Iquitos-Nauta highway, two hours of travel time resulted in a path-distance from Iquitos of 76.4 km. For the Amazon River, two hours of travel time in the fastest vehicle (a speed boat) translated into an approximate 44.5 km path-distance from Iquitos.

In all, we collected information about 48 rural communities (**Table S2.1**). Iquitos and Nauta were excluded from all analyses, as the purpose of this study was to understand *Ae. aegypti*expansion from urban to peri-urban and rural areas. We also collected information on the year of community incorporation, water system type, the number of inhabitants, and the number of houses (obtained from the 2007 Peruvian National Census) [28]. When census data were not available, population estimates were obtained from local authorities such as the mayor or the health center director.

With the exception of one site, El Terminal, all of the study sites are towns that have been officially incorporated. While El Terminal (a bus station and residential area) is not politically separated from Iquitos, it is geographically far enough to be ecologically distinct. (The distance between Iquitos and El Terminal, ~400 m, exceeds that of the estimated *Ae. aegypti* flight range, ~100 m [29]-[32].)

Entomological Data Collection

Houses were systematically selected for *Ae. aegypti* sampling and ovitrap deployment: starting at a randomly selected household within the areas of highest housing density, every Nth house was sampled based on the total number of houses in the community to ensure a minimum coverage of 10%. This resulted in a minimum of 10 and a maximum of 78 houses sampled per community.

During October through December of 2012, ovitraps were deployed in all communities along our transects. Ovitraps were red plastic cups filled ¾ water (volume = 56.5 in^3) and lined with paper. Two ovitraps were placed within each home in a dark, secluded area where they would not be a nuisance to residents. Eight days after deployment, ovitraps were checked for immature mosquitoes and removed from the household. Immature mosquitoes were collected in sterile bags (Whirlpak Co.) and transported to the field laboratory for rearing and taxonomic identification to species for *Ae. aegypti* and to genus for other mosquitoes. Paper from the ovitraps was thoroughly examined under a microscope to count the number of eggs present.*Ae. aegypti* eggs are easily differentiated from other container-breeding mosquitoes due to their smooth texture, black color, and position above the water line along the sides of containers. There are no other common container breeding *Aedes* species in this region. Although a number of *Ochleratatus* (formerly *Aedes*-genus mosquitoes) have been documented in the area [33, 34], these mosquitoes predominate in natural water bodies in forested areas such as rain pools and swamps [34–38]. *Culex* genus mosquitoes are often found in containers but they lay their eggs in rafts on the water surface. Simultaneous with ovitrap deployment, household pupal-demographic surveys were conducted to determine the abundance of wet containers and the presence of other mosquito genera in each household. Wet containers within and around each household were exhaustively surveyed for the presence of mosquito larvae. Using previously established protocols for *Ae. aegypti* surveys in Iquitos, we recorded the following information for each container: container type and material, observed solar exposure (if the container was exposed to direct sunlight at any time during the day; yes/no), degree of organic material present in the water (ranked on a scale of one to three), container location, whether the container was inside or outside or underneath a roof, whether the container was filled manually (including active collection of rain water) or passively (through unintentional rain water accumulation), and the presence of abate larvacide [39, 40]. Mosquito eggs, larvae, and pupae were collected in Whirlpak bags and were transported to the field laboratory for rearing, counting, and taxonomic identification to species for *Ae. aegypti* and to genus for other mosquitoes.

Transportation Data

River networks are the primary mode of transportation in the region (**Figure 2.1**). There are a variety of boat types that carry both passengers and cargo throughout the Peruvian Amazon including; large barges (for cargo and passengers), medium-sized barges, speed boats, and small water taxis. Terrestrial vehicle types include mini-buses taxis that travel the Iquitos-Nauta highway.

To characterize the connectivity between Iquitos and surrounding communities, 140 vehicle drivers were interviewed across 11 sites throughout Iquitos: 9 different ports and 2 bus/taxi departure points. Each sampling location was visited twice, and route information was collected for 8 taxis, 14 mini-buses, 19 medium-sized barges, 22 large barges, 25 speed boats, and 52 water taxis (a total of 140 vehicles). All available vehicle drivers were interviewed at each port or bus station. For each vehicle we collected information on the frequency and duration of travel, the final destination of the vehicle, and the number of trips to each community per month.

Data Management and Analysis

All data analysis and graphs were produced using R statistical software [41]. GPS coordinates of each town were recorded with a Garmin GPSMAP 62sc and integrated with other information (rivers, political boundaries) to create study area maps in ArcMap 10.1 (ESRI, Redlands, CA). Finalized maps were projected in Universal Transverse Mercator (UTM), Zone 18S, WGS1984 datum.

Community-Level Data

Descriptive maps of *Ae. aegypti* presence were developed by year (2008 and 2011 for the historical data, and 2011-12 for the data collected for this study). (**Table S2.2** shows datasets and data analyses employed.) A community was considered positive for *Ae. aegypti* if the mosquito was found either via ovitraps or larval surveys. For the data collected for this study (2011-12), we compared median values for community age, number of inhabitants, number of houses, and distance from the city of Iquitos (assumed to be the source population) between positive and negative communities using nonparametric Mann-Whitney Wilcoxon non-paired tests. We measured both Euclidean distance and path-distance – the latter was calculated by tracing the shortest routes from Iquitos (fluvial, terrestrial, or some combination of the two). We calculated the following entomological indices in 34 communities surveyed; Container Index (positive containers/containers surveyed *100), House Index (positive houses/houses surveyed*100), and Breteau Index (positive containers/houses surveyed *100). For one community (Varillal), we found a positive ovitrap but no positive containers from the pupal surveys.

Logistic regression models were used to explore factors associated with *Ae. aegypti* presence. The variable population size was log-transformed to force normality prior to its inclusion in the models. Other variables tested included the total wet containers and passively-filled containers, the average number of wet and passively-filled containers per house, access to the highway vs. the river, water system type, community age, the number of vehicles traveling to each location, the number of high-risk vehicles traveling to each location, and the presence of other mosquito genera. "High risk" vehicles were defined as vehicles that were most likely to contain *Ae. aegypti* mosquitoes, including large river boats for riverine communities and buses for communities along the highway. All possible combinations of variables were explored in each of the models, and the final model was selected using backwards stepwise selection in the MASS package in R [41], [42], based on the Akaike Information Criterion. Only explanatory variables that were significant univariable predictors ($p<0.10$) were included in the multivariable model. The independence of predictor variables was evaluated by testing regression models of all possible combinations of predictor variables. Residual plots were used to evaluate heteroscedasticity.

Household-Level Data

For the household-level analysis, we explored variables thought to be predictive of *Ae. aegypti*presence/absence in univariable and multivariable logistic regression models. Predictor variables included; number of people per household, the number of wet and passively-filled containers, the presence of mosquito genera within the house, and the abundance of other mosquito genera within the house.

Multivariable logistic models were developed using backwards stepwise selection in the MASS package in R [42]. The independence of predictor variables was evaluated by running regressions of all possible combinations of predictor variables

Container-Level Data

We calculated the proportion of positive containers by container type for each community positive for *Ae. aegypti* mosquitoes. To assess container productivity we calculated the proportion of pupae produced by each container type.

Univariable and multivariable logistic regression models predicting *Ae. aegypti*presence/absence were developed using the following predictor variables; container material (plastic, metal), container type (plastic bucket/pans, large drums and tanks), solar exposure (yes/no), presence or absence of a container cover, presence of other mosquito genera within the same container, the number of other mosquitoes within the same container, and fill method (manually filled vs. passively filled).

A multivariable model was developed using backwards stepwise selection in the MASS package in R [42]. Predictor variables that were correlated with one another were not included in the selection process.

2.3 Results

Community-Level Analysis

Ae. aegypti was the most abundant species, followed by *Culex*-genus mosquitoes. Other mosquito genera included; *Culex*, *Limatus*, *Toxorhynchites*, *Wyeomyia*, *Trichoprosopon*, all of which have been previously reported from the Peruvian Amazon [33, 34]. Among the 34 communities where entomological surveys were conducted for this study, 14 were positive for*Ae. aegypti* (**Figure 2.2**). Both *Ae. aegypti* and *Culex*-genus mosquitoes were found in 23.5% (8 of the 34 communities surveyed). In 17.6% (6/34) communities *Ae. aegypti* mosquitoes were found without *Culex*, and in 20.6% (7/34) of communities *Culex* mosquitoes were found without*Ae. aegypti*.

We report here the presence of *Ae. aegypti* in three new communities, two along the highway, and one along the Amazon River. Descriptive maps of *Ae. aegypti* infestation based on MOH/NAMRU collections from 2011 showed clustering of *Ae. aegypti* positive towns near Iquitos (**Figure 2.2**). Data collected for this study in 2011-12, however, showed a clear limit of *Ae. aegypti* expansion along the Iquitos-Nauta highway (Euclidean distance to Iquitos of 19.3 km). River communities, in contrast, showed a more heterogeneous spatial pattern, and the farthest point of expansion from Iquitos was 37.1 km. *Ae. aegypti* entomological indices revealed differences in mosquito abundance across sites (**Table 2.1**).

Ae. aegypti positive communities had larger population size (Mann-Whitney, U = 56, $p<0.05$), were closer to Iquitos (U = 208, $p<0.02$), and had more wet containers per household (U = 79, $p<0.05$) than *Ae. aegypti* negative communities (**Figure 2.3**). No significant differences were detected in terms of community age (U = 114, $p>0.5$) or the average number of passively-filled containers per household (U = 96, $p>0.1$).

Univariable logistic regression models (**Table S2.3)** showed that increased human population (odds ratio, OR = 1.004, $p<0.05$) and log human population (OR = 5.06, $p<0.01$) increased the odds of *Ae. aegypti* establishment. Increased Euclidean and path distance from Iquitos were both negatively associated with *Ae. aegypti* presence (OR = 0.94, $p<0.05$ for both distance measures). A higher number of wet containers resulted in an increased probability of *Ae. aegypti* establishment in that community (OR = 1.03, $p<0.05$). Since the number of wet containers per community is positively correlated with the population size (R^2 = 0.67, $p<0.0001$), we also used the average number of wet containers per household as a predictor variable. A greater number of wet containers per household increased the risk of *Ae. aegypti*establishment by 1.55 times ($p<0.05$). Communities relying predominantly on river/stream water were much less likely

(0.18 times) to have *Ae. aegypti* mosquitoes than those relying on other water sources (e.g., well water, piped water) (p<0.05). Variables with no significant impact on*Ae. aegypti* presence included; community age, the number of vehicles (and high risk vehicles) traveling to each site per month, the average number of passively-filled containers/household, the absolute number of passively-filled containers per household, use of piped water, access to the Amazon River, and presence of other mosquito genera.

For the multivariable model the log-transformed human population number (which had a more powerful effect than the raw population number) was used. Euclidean distance was used because it had a lower AIC value than path distance from Iquitos. The predictor variables included in the model selection process were; log human population, Euclidean distance from Iquitos, the average number of wet containers per household, and whether or not the community relied on river or stream water. The final multivariable logistic regression model (**Table 2.2**) showed that the risk of *Ae. aegypti* establishment is increased 5.76 times per log population unit (p<0.05), when taking into account the Euclidean distance from Iquitos and the use of river/stream water. Communities farther away from Iquitos were less likely to have *Ae. aegypti* mosquitoes (OR = 0.89, p<0.05) when adjusting for the other variables included in the model. The use of river/stream water was not statistically significant.

House-Level Analysis

Of the 580 houses (in 34 communities) that we surveyed, 80 (13.8%) were positive for *Ae. aegypti*. Among houses (N = 380) in the 14 positive communities, 22.9% houses were positive (80/350 houses). House-level logistic regression models were restricted to communities in which *Ae. aegypti* was present. In houses in positive communities, *Culex* mosquitoes were found together with *Ae. Aegypti* in 4.2% (16/380) of houses. In 16.6% (63/380) of houses in positive communities, *Ae. aegypti* were found without *Culex*, and in 2.6% (10/380) houses*Culex* mosquitoes were present without *Ae. aegypti*.

Univariable logistic regression (**Table S2.4**) showed that the number of wet containers found within a household slightly increased the risk of *Ae. aegypti* presence (OR = 1.05, p<0.05). Houses with higher number of passively-filled containers were 1.17 times more likely to have*Ae. aegypti* mosquitoes (p<0.001). Both the presence and number of other mosquitoes within the household increased the risk of *Ae. aegypti* presence (OR = 5.85, p<0.001, and OR = 5.44, p <0.001, respectively).

Given that the numbers of wet and passively-filled containers in a house are correlated, we chose to include only the number of passively-filled containers in the multivariable model, as the AIC and p-value were both lower. Similarly, the number of other mosquitoes was chosen over the presence/absence of other mosquitoes, based on the AIC and significance levels of the univariable predictors. The final multivariable logistic regression model (**Table 2.3**) showed that the number of passively-filled containers increased risk of *Ae. aegypti* presence 1.16 times (p<0.001), while the presence mosquitoes of other genera present increased this risk 5.67 times (p<0.001).

Container-Level Analysis

Among containers that were positive for *Ae. aegypti*, *Culex* genus mosquitoes were also found in 8.6% of containers (11/128). The most common types of water-holding containers found (regardless of infestation status) were plastic buckets (61.2% of all containers) and large water drums (10.5% of all containers). Although plastic containers were very common (1,977 found), the proportion infested was small (2.7%). Toilets and drains had the highest infestation level (17.0% positive of 23), followed by tires (12.8% positive of 39) and large water storage tanks/drums (11.5% positive of 340) (**Table 2.4**). Productivity analysis by container type (**Figure 2.4**) demonstrated that plastic containers and water storage tanks/drums produced 41.1% and 35.6% of all pupae, respectively, followed by animal watering pans(11.7%). A similar pattern held for larval productivity, with plastic containers and water tanks/drums accounting for 33.4% and 32.0% of the larvae, respectively.

2.4 Discussion

To our knowledge this is the first extensive, multi-scale analysis of *Ae. aegypti* geographic expansion from urban to peri-urban and rural areas. Of the three ecological scales, the most novel findings were based on our community-level data, as the house and container-level data simply confirmed previous findings about *Ae. aegypti*. At the container-level, for example, water tanks/drums have previously been shown to be important for *Ae. aegypti* production, and *Ae. aegypti* has been shown to co-exist with *Culex* genus mosquitoes [40]. (The co-occurrence of*Ae. aegypti* and *Culex* mosquitoes in households is likely attributable to the abundance of suitable containers that are favorable to all container-breeding mosquitoes, and the availability of shade and sufficient organic material for larval feeding.) Below we discuss further these effects of water use, population size, and distance from Iquitos on the invasion process. We also explore the spatial pattern of *Ae. aegypti* spread, and its implications for DENV transmission.

Community use of river/stream water reduced the odds of *Ae. aegypti* establishment. While this variable was not statistically significant in our multivariable model, we suspect that this may be due to relatively small number of observations and low statistical power. The observed association may be a result one of three mechanisms 1) Water use may be correlated with other factors important for *Ae. aegypti* establishment and spread. (For example, piped water systems are likely to be most abundant in larger settlements closer to Iquitos city.) 2) River/stream water may be less attractive to *Ae. aegypti* mosquitoes for oviposition due to the chemical and organic composition of the water. 3) Containers filled with river/stream water may be frequently emptied and re-filled, thus reducing the probability of the accumulation of organic material and therefore oviposition. For the first mechanism, we were unable to identify a significant correlation between river/stream water usage and population size or distance to Iquitos (**Figure S2.1**). The second mechanism could be evaluated through simple oviposition experiments to test the hypothesis that river water is less suitable for *Ae. aegypti* oviposition and development, similar to that in [43, 44]. Lastly, longitudinal studies could elucidate patterns of container use and quantify water turnover [45] by water source in rural areas, although this is likely to vary depending on local cultural and socioeconomic conditions.

Human population size may influence *Ae. aegypti* invasion in two ways. First, large population centers are more likely to have an abundance of oviposition sites, thus contributing to greater habitat suitability. Secondly, population centers are also more “connected” to other places via vehicle traffic, thereby contributing to human-mediated introduction of immature mosquitoes (introductory pressure). Thus, from an invasion ecology perspective, we cannot disentangle the effects of habitat suitability vs. introductory pressure, since both are correlated with population size. Regardless of which of these individual mechanisms (or combination of the two) is driving the observed associations, *Ae. aegypti,* control programs should address both habitat suitability (wet container management/reduction, insecticide spraying) and introductory pressure (surveillance and control of mosquitoes on vehicles).

Our analyses revealed that population and inverse distance increased risk of *Ae. aegypti*presence at a community scale. While beyond the scope of this paper, gravity models may be an appropriate way to predict the future spread of *Ae. aegypti* mosquitoes. Although gravity models have been used to model

DENV dispersal [46, 47], to date, such an approach has not been applied to *Ae. aegypti* spread. Gravity models assume that connectivity between locations is a function of the inverse distance between them and of their ‘attractiveness’ based on population size. They have been applied to invasive organisms that spread through human-mediated activities [48–50]. A key advantage to this approach is that the required data are often easily accessible through public resources such as census data and public maps.

The contrast between the spatial pattern of *Ae. aegypti* establishment along rivers vs. the highway is noteworthy. Genetic studies have suggested that *Ae. aegypti* spreads along transportation networks [51–53], but few studies to date have used field collections to identify areas where *Ae. aegypti* has become successfully established following introduction from a known source [13, 14, 54]. We propose that urbanization is responsible for the linear pattern observed along the highway, due to the high density of settlements relatively close to Iquitos and immediately adjacent to the highway. Short-distance active dispersal of *Ae. aegypti* mosquitoes is driven by availability of oviposition sites [32], and as urbanization continues southward, ovisposition sites become more abundant. The community 5 de Abril represents the geographic limit of *Ae. aegypti* along the highway, approximately 19 km south of Iquitos. This is most likely due to the ~6.5 km gap between 5 de Abril and the next community to the south, San José (**Figure 2.5**). Prior to that point, each community is distanced <3.1 km from the next settlement along the highway.

In contrast, it is probable that *Ae. aegypti* geographic expansion along fluvial routes is the result of longer-distance dispersal events that are mediated via the passive transport of mosquito eggs through human vehicles (boats). Urbanization along the highway is more relevant for DENV transmission, owing to greater density of human host and vector populations [55, 56]. Mahabir et al (2012) showed that arterial highways in Trinidad tended to have fewer dengue cases than did smaller, rural roads, as major highways may serve as barriers to transmission [57]. With only one lane in each direction, the Iquitos-Nauta highway would likely be classified as a smaller road leading to rural towns. Thus, we believe that as urbanization south of Iquitos continues, the conditions along the Iquitos-Nauta highway will grow increasingly suitable for DENV transmission. In contrast, while riverine communities may be susceptible to mosquito introductions, most of these towns currently lack enough human hosts to ensure sustained local DENV transmission. (Realistic estimates of minimum human population required for local transmission range from 3,000 to 100,000 [58–61].)

Our approach may be applicable to *Ae. aegypti* in other regions (e.g., Vietnam's Mekong Delta which relies heavily on fluvial transport), and to other insect vectors that are passively transported by humans (e.g., *Aedes albopictus* [62], *Culex quinquefasciatus* [63]; and *Triatoma infestans* [64], among others [65]).

2.5 Tables

Table 2.1. Entomological Indices for communities positive for Ae. aegypti (collected data only). Indices quantifying *Ae. aegypti* densities include; Container Index (positive containers/ containers surveyed *100), House Index (positive houses/ houses surveyed*100), and Breteau Index (positive containers/ houses surveyed *100).

Community	**Container Index (CI)**			**House Index (HI)**		
	Positive containers	**Containers surveyed**	**CI**	**Positive houses**	**Houses surveyed**	**HI**
Aucayo	27	110	**24.55**	12	15	**80.00**
Nueva Unión	11	73	**15.07**	6	10	**60.00**
25 de Enero	6	88	**6.82**	4	11	**36.36**
Peña Negra	8	157	**5.10**	5	14	**35.71**
Barrio Florida	7	141	**4.96**	5	16	**31.25**
Cruz del Sur	3	63	**4.76**	3	13	**23.08**
Los Delfines	17	331	**5.14**	10	45	**22.22**
El Terminal	2	73	**2.74**	2	10	**20.00**
Quistococha	16	338	**4.73**	8	41	**19.51**
Tamshiyacu	17	556	**3.06**	12	78	**15.38**
Indiana	13	909	**1.43**	10	76	**13.16**
Santa Clotilde	1	77	**1.30**	1	9	**11.11**

5 de Abril	1	60	**1.67**	1	11	**9.09**

Table 2.1 continued

Community	Breteau Index (BI)		
	Positive containers	**Houses surveyed**	**BI**
Aucayo	27	15	**180.00**
Nueva Unión	11	10	**110.00**
25 de Enero	6	11	**54.55**
Peña Negra	8	14	**57.14**
Barrio Florida	7	16	**43.75**
Cruz del Sur	3	13	**23.08**
Los Delfines	17	45	**37.78**
El Terminal	2	10	**20.00**
Quistococha	16	41	**39.02**
Tamshiyacu	17	78	**21.79**
Indiana	13	76	**17.11**
Santa Clotilde	1	9	**11.11**
5 de Abril	1	11	**9.09**

Table 2.2. Multivariable logistic regressions: Ae. aegypti risk factors at the community scale. Statistically significant ($p<0.05$) variables are shown in bold (N=34 communities).

Variable	OR	95% CI	SE	P
Log(Population)	**5.76**	**1.78, 34.49**	**0.72**	**<0.05**
Euclidean dist. from IQT (km)	**0.89**	**0.76, 0.97**	**0.058**	**<0.05**
River/ stream water	0.094	0.0046, 0.90	1.30	>0.05

Table 2.3. Multivariable logistic regressions: Ae. aegypti risk factors at the house scale. Statistically significant ($p<0.05$) variables are shown in bold (N=380 houses).

Variable	OR	95% CI	SE	P
No. passively-filled containers	**1.16**	**1.08, 1.27**	**0.04**	**<0.001**
Presence of mosquitoes of other genera	**5.67**	**2.54, 13.05**	**0.41**	**<0.001**

Table 2.4. Proportion of positive containers by type. A container was considered positive for *Ae. aegypti* mosquitoes if it had larvae or pupae. (The table below does not include two containers that had eggs, but no larvae or pupae.)

Container Type	**Number positive**	**Number negative**	**Total Found**	**Percent Positive**
Toilet/ drains	4	19	23	17.39
Tires	5	34	39	12.82
Water storage tanks/ drums	39	301	340	11.47
Animal watering pans/ fish ponds	7	103	110	6.36
Nontraditional (puddles, plants)	3	45	48	6.25
Trash (discarded items)	11	342	353	3.12
Plastic containers (buckets, pans)	54	1923	1977	2.73
Dishes (plates, mugs, plate holders)	5	310	315	1.59
Wells	0	28	28	0.00
Total	**128**	**3105**	**3233**	**3.96**

Table 2.5. Multivariable logistic regressions: Ae. aegypti risk factors at the container scale. Variables included in the multivariable model selection processes were; type= drums/ tanks, presence of mosquitoes of other genera, solar exposure, and presence of a container lid. Statistically significant ($p<0.05$) variables are shown in bold (N=3235 containers).

Variable	**OR**	**95% CI**	**SE**	**P**
Type= drum/tank	4.22	2.81, 6.23	0.20	<0.0001
Solar Exposure= yes	2.53	1.72, 3.81	0.20	<0.0001

2.6 Figures

Figure 2.1. Map of study area. Iquitos is the largest city in the Peruvian Amazon (pop: 380,000), and is accessible only by boat or plane. There are approximately 500,000 people living in the study area shown on the right side of the map. Fluvial routes are the predominant mode of transportation in the region.

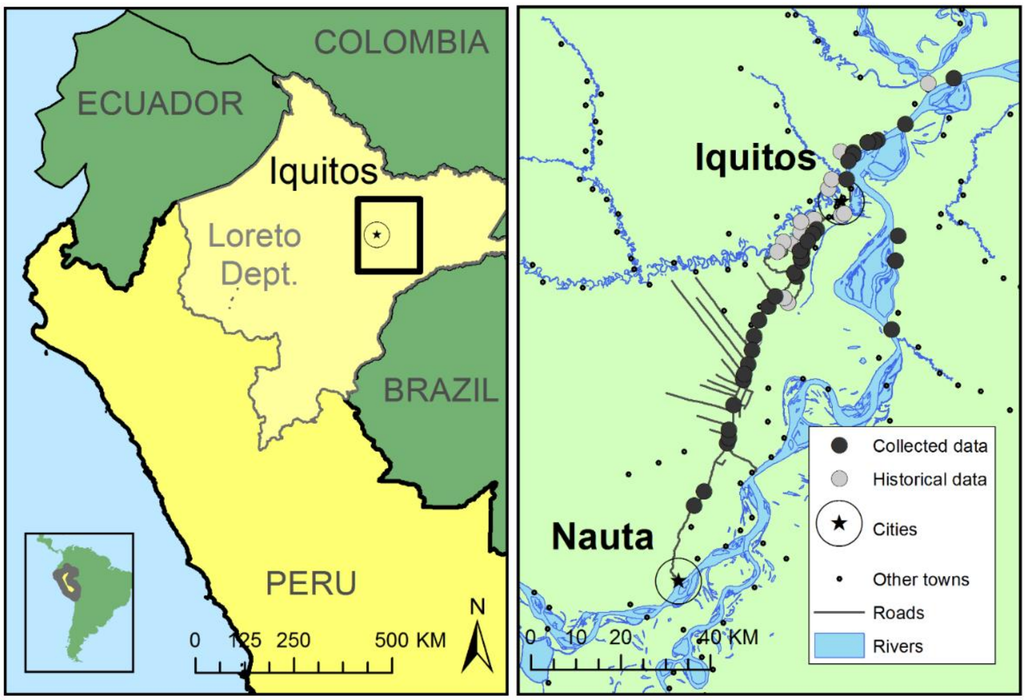


Figure 2.2. Ae. aegypti presence-absence by data source. Results from pupal demographic surveys/ ovitraps are shown for A) Historical data from MOH/ NAMRU data in 2008, B) Historical data from MOH/ NAMRU in 2011 , and C) Data collected for this study in 2011-12(N=34).

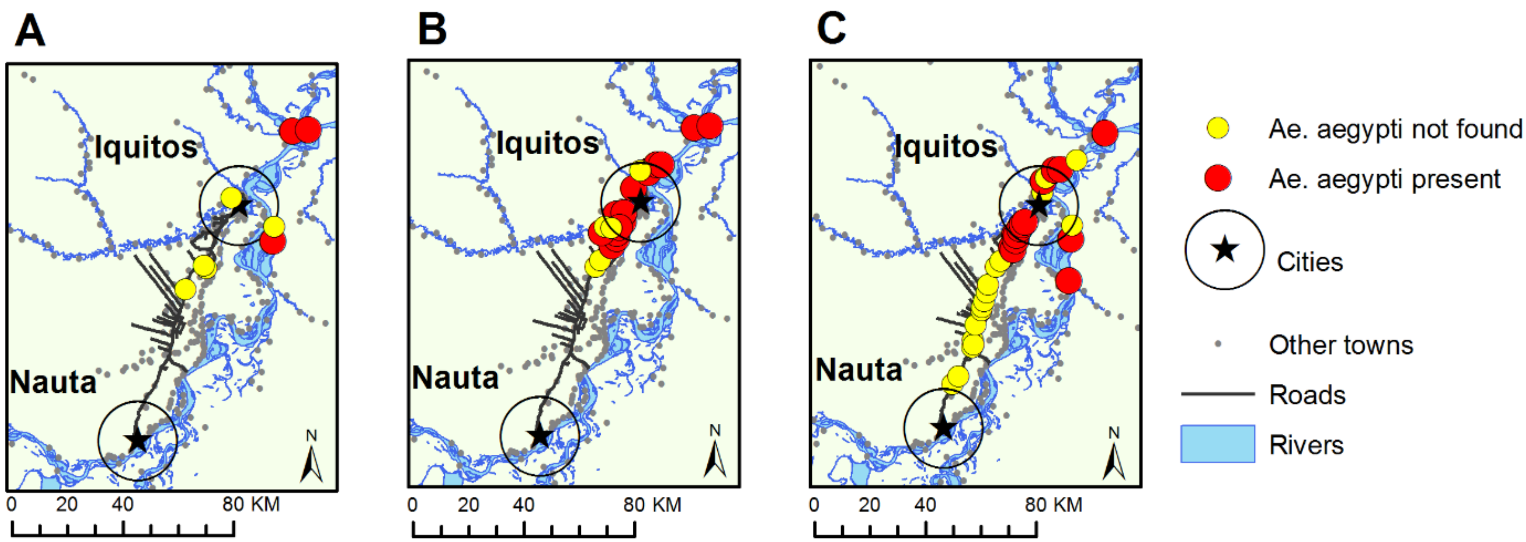


Figure 2.3. Mann-Whitney Wilcoxon tests for median differences in Ae. aegypti positive vs. negative communities. Significant differences ($p<0.05$) between *Ae. aegypti* positive vs. negative communities were detected in terms of human population size, distance from Iquitos, and the number of wet containers per house.

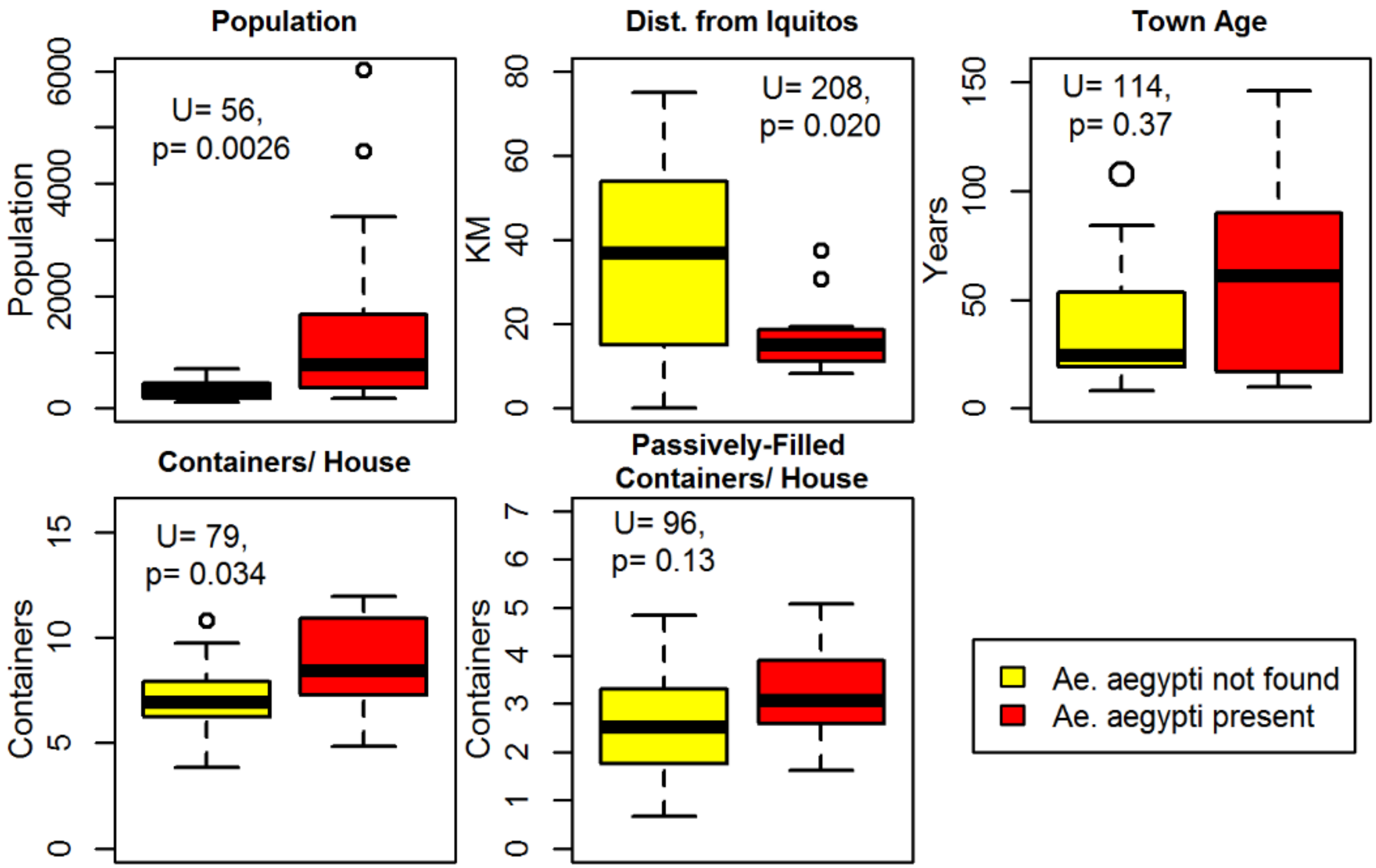


Figure 2.4. Laval and pupal productivity by container type. A) Larval productivity, B) Pupal productivity.

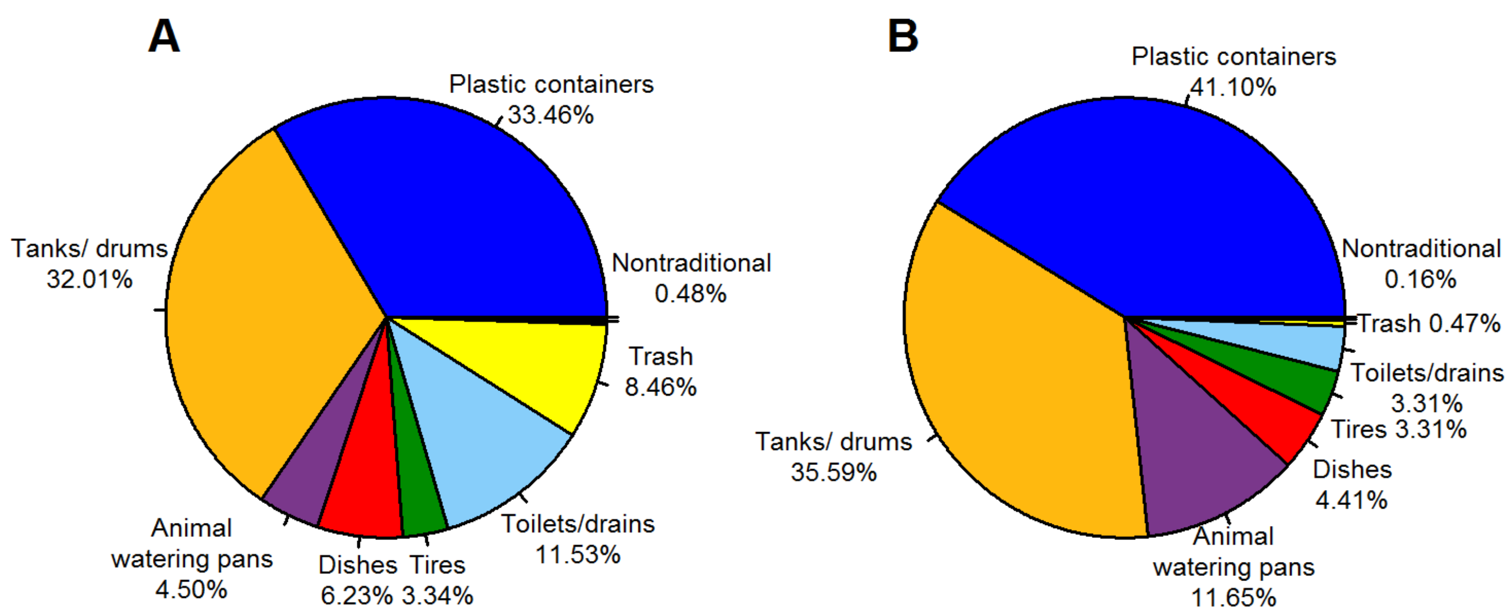
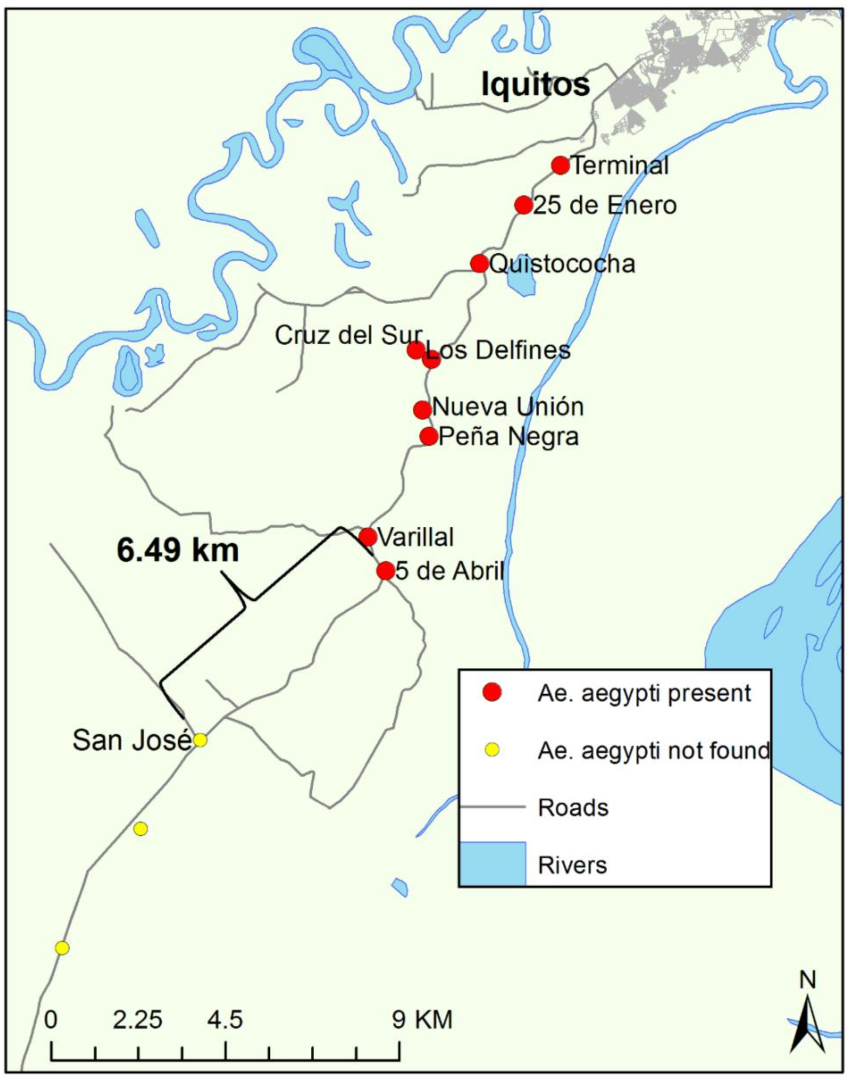


Figure 2.5. Geographic border of Ae. aegypti colonization along the Iquitos-Nauta highway. The distance between the southernmost positive community, 5 de Abril, and the next community, San José, is approximately 6.49km. The space between these two communities is characterized by forest cover with no human settlements (and therefore no oviposition sites). This forested area likely acts as a barrier to *Ae. aegypti* dispersal.



2.7 Supplementary Tables

Table S2.1. Characteristics of communities included in the study. + indicates that *Ae. aegypti* was found, - indicates that *Ae. aegypti* was not found. A blank space indicates that data were not collected for that community.

Community	Houses	Inhabitants	Year of incorporation
Iquitos	~81923*	406340*	1866
Nauta	2741	13983	1830
El Terminal	1444***	6036***	1866
Tamshiyacu	1039	4583	1883
Indiana	748	3410	1948
Mazan	653	3184	1943
Santa Clara de Nanay	625	2868	1933
Rumococha	518*	4341*	1912
Los Delfines	459	1679	2000
Quistococha	402	1496	1909
Varillal	371*	1050*	1949
Padre Cocha	323	1627	1917
Santo Tomás	302	1308	1866
31 de Mayo	287**	600**	2004
Cahuide	167	703	1965
Barrio Florida	157	737	1953
Zungaro Cocha	155	782	1965
Aucayo	153	806	1922
Nina Rumi	139	561	1928
Manacamiri	136	682	1913
13 de Febrero	131	473	1990
Nuevo Horizonte	131	452	1985
Cruz del Sur	128	455	1987
Gallito	125	673	1936
Santa María de Ojeal	121	550	1928
Peña Negra	121	520	1942
San Lucas	116	450	1991
Ex-Petroleros	111	412	1986
Santa Clara de Ojeal	106*	281*	1952
Sinchicuy	105	449	1904
El Dorado	97	312	1999
Laguna Azul	96*	420*	2004
El Triunfo	94	319	1989
25 de Enero	84	364	1992
Picuroyacu	82	355	1930
1 de Febrero	76	290	2004
Nuevo Milagro	68	241	1996
Buen Pastor	66	238	1977
12 de Abril	63	198	1990
Santa Clotilde	58	255	1995
Nueva Unión	57	197	2002
Puerto Alemendras	54	205	1917
Santa Clara de Ojeal III	52	230	1990
El Paujil	50	185	1985
La Habana	43	155	1986

5 de Abril	38**	180**	1998
San José	34	118	1997
Independencia	32	106	1995
Nuevo San Juan	30	148	2000
Lupunillo	18	101	1932

Table S2.1 continued

Community	Data Source=MOH	Data Source=NAMRU	Data Source=Collected	Year(s) of collections
Iquitos	+	+		Ongoing
Nauta	+	+		Ongoing
El Terminal			+	2012
Tamshiyacu		+	+	2008, 2012
Indiana	+	+	+	2008, 2012
Mazan	+	+		2008, 2010, 2011, 2012
Santa Clara de Nanay	+			2011
Rumococha	+			2011
Los Delfines	+		+	2011, 2012
Quistococha	+		+	2011, 2012
Varillal	+		+	2012
Padre Cocha	+	+		2011
Santo Tomás	+			2011
31 de Mayo	+			2011
Cahuide			-	2012
Barrio Florida	+	+	+	2011, 2012
Zungaro Cocha	-	-		2011
Aucayo	+	+	+	2008, 2012
Nina Rumi	-	-		2011
Manacamiri	-			2008
13 de Febrero		-	-	2008
Nuevo Horizonte			-	2012
Cruz del Sur	+		+	2011, 2012
Gallito		-	-	2008, 2012
Santa María de Ojeal	+		-	2011, 2012
Peña Negra	+		+	2011, 2012
San Lucas			-	2012
Ex-Petroleros			-	2012
Santa Clara de Ojeal	+		-	2011, 2012
Sinchicuy			-	2012
El Dorado	-		-	2011
Laguna Azul	+			2011
El Triunfo			-	2012
25 de Enero	+		+	2011, 2012
Picuroyacu	-			2011
1 de Febrero			-	2012
Nuevo Milagro	-			2008
Buen Pastor		-		2008
12 de Abril			-	2012
Santa Clotilde			+	2012
Nueva Unión	+		+	2011, 2012
Puerto Alemendras	-			2011
Santa Clara de Ojeal III			-	2012
El Paujil			-	2012
La Habana			-	2012
5 de Abril			+	2012
San José	-		-	2011, 2012
Independencia	+		-	2011, 2012
Nuevo San Juan			-	2012
Lupunillo			-	2012

*Source: Government health center (2012)
**Source: Interview with lieutenant governor (2012)
***Source: Number of houses estimated from Google Earth imagery (Iquitos, Peru, lat -3798893° lon -

73.308773°, DigitalGlobe, Landsat, US Geological Survey: Google Earth), and population was estimated by calculating the average number of people per home for the other communities (4.18) and extrapolating.

Table S2.2. Datasets, ecological scales, and statistical analyses employed.

	Historical Data (NAMRU + MOH)	Collected Data		
Entomological Data	*Ae. aegypti* presence-absence	*Ae. aegypti* presence-absence		
Scale of Data	Community	Community	House	Container
Analyses	• Descriptive maps	• Entomological Indices • Mann-Whitney Wilcoxon tests • Logistic regression	• Logistic regression	• Descriptive statistics • Logistic regression

Table S2.3. Community-level univariable logistic regression models.

Statistically significant (p<0.05) variables are shown in bold. Variables were included in the multivariate selection process with an entry criterion of p<0.10.

Model	Variable	OR	95% CI	SE	P	AIC
1	**No. wet containers**	**1.032**	**1.00025, 1.064**	**0.016**	**<0.05**	**35.65**
2	**Population**	**1.0036**	**1.00026, 1.0070**	**0.0017**	**<0.05**	**36.54**
3	No. rain-filled containers	1.07	0.9952, 1.14	0.036	<0.05	36.63
4	**Log(Population)**	**5.06**	**1.88, 13.59**	**0.63**	**<0.05**	**38.15**
5	**Euclidean dist. from IQT (km)**	**0.94**	**0.88, 0.99**	**0.029**	**<0.05**	**41.52**
6	**Path dist. from IQT (km)**	**0.94**	**0.90, 0.99**	**0.025**	**<0.05**	**42.13**
7	**River/ stream water (vs. other types)**	**0.18**	**0.038,0.86**	**0.80**	**<0.05**	**44.88**
8	**Avg. wet containers/ house**	**1.55**	**1.0016, 2.40**	**0.22**	**<0.05**	**45.45**
9	Age of town (years)	1.014	0.99, 1.04	0.011	>0.1	46.61
10	Avg. rain-filled containers/ house	1.60	0.83, 3.094	0.34	>0.1	47.92
11	Potable water (vs. other types)	3.60	0.56, 23.24	0.95	>0.1	48.14
12	No. vehicles/ month	0.9989	0.99,1.0011	0.0011	>0.1	49.06
13	No. high-risk vehicles	0.9984	0.9951, 1.0017	0.0017	>0.1	49.15
14	Presence of competitors	0.6	0.13, 2.71	0.76	>0.1	49.62
15	Amazon River access (vs. road)	1.032	0.26 , 4.30	0.73	>0.1	50.07

Table S2.4. House-level univariable logistic regression models. Statistically significant (p<0.05) variables are shown in bold. Variables were included in the multivariate selection process with an entry criterion of p<0.10.

Model	Variable	OR	95% CI	SE	P	AIC
1	**No. competitor mosquitoes present**	**5.44**	**2.64, 11.96**	**0.38**	**<0.001**	**354.50**
2	**Presence of competitors**	**5.85**	**2.68, 13.17**	**0.403**	**<0.001**	**357.10**
3	**No. rain-filled containers**	**1.17**	**1.083, 1.27**	**0.0404**	**<0.001**	**360.50**
4	**No. wet containers**	**1.053**	**1.0061, 1.103**	**0.023**	**<0.05**	**371.40**
5	Inhabitants/ household	1.12	0.99, 1.25	0.057	>0.05	372.60

Table S2.5. Container-level univariable logistic regression models.

Statistically significant (p<0.05) variables are shown in bold. Variables were included in the multivariate selection process with an entry criterion of p<0.10.

Model	Variable	OR	95% CI	SE	P	AIC
1	Fill method = rain	0.092	0.0036, 2.33	1.418	>0.05	997.36
2	**Presence of competitors**	**12.60**	**6.96, 22.09**	**0.29**	**<0.001**	**1035.50**
3	**Type = drum/ tank**	**4.04**	**2.70, 5.95**	**0.20**	**<0.001**	**1048.20**
4	**Container lid = yes**	**0.15**	**0.060, 0.32**	**0.42**	**<0.01**	**1053.00**
5	**Solar exposure = yes**	**2.42**	**1.65, 3.64**	**0.20**	**<0.001**	**1066.60**
6	**Type = plastic container**	**0.44**	**0.31, 0.63**	**0.18**	**<0.001**	**1068**
7	**Type = toilet/ drain**	**5.24**	**1.50,14.19**	**0.56**	**<0.01**	**1075.30**
8	**Type = tire**	**3.64**	**1.23, 8.68**	**0.49**	**<0.01**	**1082.80**
9	Type = animal watering pan	1.67	0.69, 3.43	0.40	>0.10	1086.60
10	Type = nontraditional	1.071	0.17, 3.52	0.73	>0.10	1088
11	Material = Metal	1.40	0.80, 2.30	0.27	>0.10	1093.40
12	Material = Plant	1.14	0.063, 5.51	1.027	>0.10	1094.80

Table S2.6. Container-level univariable logistic regression models demonstrating multicollinearity among all possible combinations of predictor variables. The tables below demonstrate logistic regression models using all possible combinations of predictor variables. Significant ($p<0.05$) predictor variables are shown in bold.

A **Outcome: Presence of competitors**

Model	**Variable**	**OR**	**95% CI**	**SE**	**P**	**AIC**
1	**Container lid = yes**	**0.17**	**0.041, 0.45**	**0.59**	**<0.005**	**593.55**
2	**Type = plastic container**	**0.41**	**0.24, 0.68**	**0.26**	**<0.001**	**597.41**
3	**Type =drum/tank**	**2.60**	**1.37, 4.65**	**0.31**	**<0.005**	**601.30**
4	**Solar Exposure = yes**	**2.11**	**1.23, 3.77**	**0.28**	**<0.01**	**601.71**
5	**Type = tire**	**6.29**	**1.84, 16.42**	**0.54**	**<0.001**	**601.80**
6	Type = toilet/drain	5.089	0.80, 17.90	0.75	<0.05	606.16

B **Outcome: drum/ tank**

Model	**Variable**	**OR**	**95% CI**	**SE**	**P**	**AIC**
1	Type = plastic container	$3.12 *10^{-09}$	$3.32*10^{-84}$,$1.38*10^{-138}$	399.066	>0.05	1474.00
2	**Container lid = yes**	**1.68**	**1.31, 2.14**	**0.12**	**<0.0001**	**2162.40**
3	Type = tire	$5.37*10^{-07}$	$9.74*10^{-60}$,18.56	384.23	>0.05	2170.20
4	Type = toilet/ drain	$1.47*10^{-06}$	NA, 26.62	303.47	>0.05	2173.70
5	Solar Exposure = yes	0.88	0.70, 1.10	0.11	>0.10	2177.70

C **Outcome: Container lid**

Model	Variable	OR	95% CI	SE	P	AIC
1	**Solar Exposure = yes**	**0.049**	**0.038, 0.064**	**0.13**	**<0.0001**	**2654.70**
2	**Type = plastic container**	**2.41**	**2.0045, 2.90**	**0.09**	**<0.0001**	**3417.80**
3	Type = tire	$5.64*10^{-07}$	$5.90*10^{-39}$, 0.021	233.0501	>0.05	3491.40
4	Type = toilet/ drain	0.15	0.0083, 0.71	1.023	>0.05	3505.90

D **Outcome: Solar exposure**

Model	Variable	OR	95% CI	SE	P	AIC
1	**Type = plastic container**	**0.38**	**0.33, 0.44**	**0.075**	**<0.0001**	**4300.30**
2	**Type = toilet/ drain**	**4.18**	**1.57, 14.45**	**0.55**	**<0.01**	**4465.10**
3	**Type = tire**	**6.037**	**2.58, 17.65**	**0.48**	**<0.0005**	**4453.20**

E **Outcome: Plastic container**

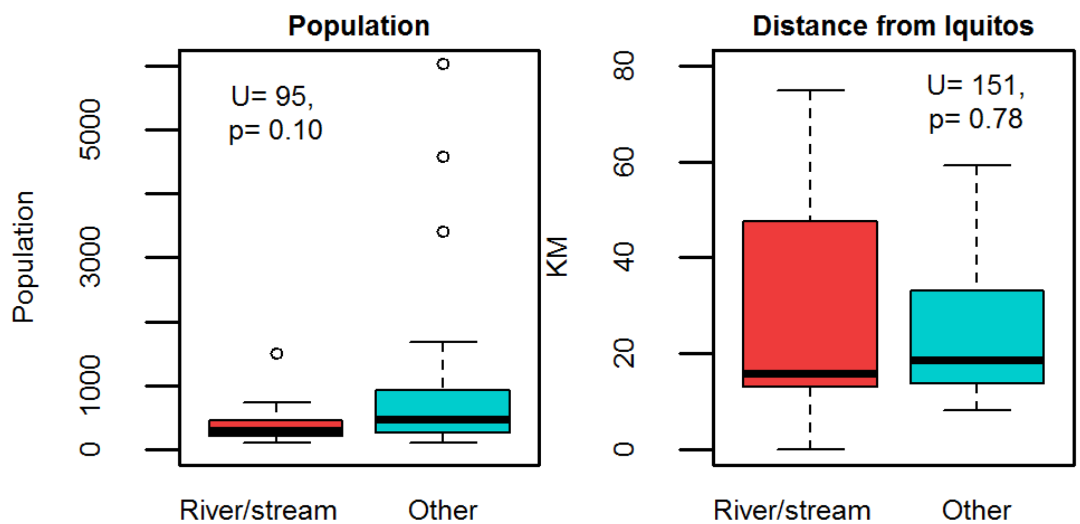
Model	Variable	OR	95% CI	SE	P	AIC
1	Type = Toilet/ drain	$2.96*10^{-07}$	NA, 0.0075	184.065	>0.05	4286.60
2	Type = tire	$1.075*10^{-07}$	$1.13*10^{-39}$, 0.0040	233.05	>0.05	4256

F **Outcome: Toilet/ drain**

Model	Variable	OR	95% CI	SE	P	AIC
1	Type = tire	$1.19*10^{-06}$	NA, 7.73	1044.46	>0.05	276.81

2.8 Supplementary Figures

Figure S2.1. River/ stream water vs. other water types by population and distance from Iquitos. Mann-Whitney Wilcoxon tests showed no significant correlation between river/ stream water usage and population size or distance to Iquitos.



2.9 References Cited

1. World Health Organization (2012) Dengue and severe dengue. Available: http://www.who.int/mediacentre/factsheets/fs117/en/. Accessed: May 18, 2013.

2. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2013) The global distribution and burden of dengue. Nature 496: 504-507.

3. Halstead SB (2008) Dengue virus-mosquito interactions. Annu Rev Entomol 53: 273-291.

4. Centers for Disease Control and Prevention (2013) Dengue Entomology and Ecology: Mosquitoes' Main Aquatic Habitats. Available: www.cdc.gov/dengue/entomologyEcology/m_habitats.html. Accessed: May 18, 2013.

5. Sota T, Mogi M (1992) Interspecific variation in desiccation survival time of *Aedes* (Stegomyia) mosquito eggs is correlated with habitat and egg size. Oecologia 90: 353-358.

6. Juliano SA, O'Meara GF, Morrill JR, Cutwa MM (2002) Desiccation and thermal tolerance of eggs and the coexistence of competing mosquitoes. Oecologia 130: 458-469.

7. Soper FL (1967) Dynamics of *Aedes aegypti* distribution and density. Seasonal fluctuations in the Americas. Bull World Health Organ 36: 536-538.

8. Gubler DJ (1997) Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. Dengue and dengue hemorrhagic fever. Cambridge, UK CAB International pp. 1-22.

9. Pan American Health Organization (1986) *Aedes aegypti*: Biology and Ecology. Nelson, MJ, editor. Washington, DC.

10. Schliessman DJ, Calheiros LB (1974) A review of the status of yellow fever and *Aedes aegypti* eradication programs in the Americas. Mosq News 34: 1-9.

11. Soper FL, Wilson DB, Lima S, Antunes W (1943) The organization of permanent nationwide anti-*Aedes aegypti* measures in Brazil. New York: The Rockefeller Foundation.

12. Gubler DJ (1989) *Aedes aegypti* and *Aedes aegypti*-borne disease control in the 1990s: top down or bottom up. Am J Trop Med Hyg 40: 571-578.

13. Diaz-Nieto LM, Macia A, Perotti MA, Beron CM (2013) Geographical limits of the Southeastern distribution of *Aedes aegypti* (Diptera, Culicidae) in Argentina. PLoS Negl Trop Dis 7: e1963.

14. Troyes L, Villegas Z, Troyes M (2006) Expansión del *Aedes aegypti* a localidades rurales de Cajamarca. Rev Perú Med Exp Salud Pública 23: 163-167.

15. Phillips I, Need J, Escamilla J, Colan E, Sanchez S, et al. (1992) First documented outbreak of dengue in the Peruvian Amazon region. Bull Pan Am Health Organ 26: 201-207.

16. Chapple DG, Simmonds SM, Wong BBM (2012) Can behavioral and personality traits influence the success of unintentional species introductions? Trends Ecol Evol 27: 57-64.

17. Lockwood JL, Cassey P, Blackburn TM (2009) The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. Divers Distrib 15: 904-910.

18. Williamson MH (1996) Biological invasions. London; New York: Chapman & Hall.

19. Burke MJW, Grime JP (1996) An Experimental Study of Plant Community Invasibility. Ecology 77: 776-790.

20. Moyle PB, Light T (1996) Biological invasions of fresh water: Empirical rules and assembly theory. Biol Cons 78: 149-161.

21. Holway DA (1998) Factors governing rate of invasion: a natural experiment using Argentine ants. Oecologia 115: 206-212.

22. Levine JMDA, Carla M. (1999) Elton revisited: a review of evidence linking diversity and invasibility. Oikos 87: 15-26.

23. Gomez Romero ETO, T, (1998) Uso de la tierra y patrones de deforestación en la zona de Iquitos. In: Kalliola R FPS, editor. Geoecología y desarrollo amazónico: estudio integrado en la zona de Iquitos, Perú Sulkava: Finnreklama Oy. pp. 369-388.

24. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, et al. (2006) The effect of deforestation on the human-biting rate of Anopheles darlingi, the

primary vector of Falciparum malaria in the Peruvian Amazon. Am J Trop Med Hyg 74: 3-11.

25. Kalliola RF-P, S (1998) Geoecología y desarolla amazónico: estudio integrado en la zona de Iquitos. Annales Universitatis Turkuensis Ser A II. Turku, Finland: Turku University. pp. 544.
26. Ministerio de Salud del Perú, Dirección General de Salud Ambiental (2011) Norma técnica de salud para la implementatción de la vigilancia y control del *Aedes aegypti*, vector del dengue en el territorio nacional. Lima, Peru.
27. Vazquez-Prokopec GM, Galvin WA, Kelly R, Kitron U (2009) A new, cost-effective, battery-powered aspirator for adult mosquito collections. J Med Entomol 46: 1256-1259.
28. Instituto Nacional de Estadística e Informática (2007) Censo peruano. Lima, Peru.
29. Morlan HB, Hayes RO (1958) Urban dispersal and activity of *Aedes aegypti*. Mosq News: 137-144.
30. Muir LE, Kay BH (1998) *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. Am J Trop Med Hyg 58: 277-282.
31. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg 72: 209-220.

32. Edman JD, Scott TW, Costero T, Morrison AC, Harrington LC, et al. (1998) *Aedes aegypti* (Diptera: Culicidae) movement influenced by availability of oviposition sites. J Med Entomol 35: 578-583.

33. Johnson MF, Gomez A, Pinedo-Vasquez M (2008) Land use and mosquito diversity in the Peruvian Amazon. J Med Entomol 45: 1023-1030.

34. Pecor JE, Jones J, Turell MJ, Fernandez R, Carbajal F, et al. (2000) Annotated checklist of the mosquito species encountered during arboviral studies in Iquitos, Peru (Diptera: Culicidae). J Am Mosq Control Assoc 16: 210-218.

35. Alfonzo D, Grillet ME, Liria J, Navarro JC, Weaver SC, et al. (2005) Ecological characterization of the aquatic habitats of mosquitoes (Diptera: Culicidae) in enzootic foci of Venezuelan equine encephalitis virus in western Venezuela. J Med Entomol 42: 278-284.

36. Cardoso Jda C, de Paula MB, Fernandes A, dos Santos E, de Almeida MA, et al. (2011) Ecological aspects of mosquitoes (Diptera: Culicidae) in an Atlantic forest area on the north coast of Rio Grande do Sul State, Brazil. J Vector Ecol 36: 175-186.

37. Kumm HW, Komp WHW, Ruiz H (1940) The mosquitoes of Costa Rica. Am J Trop Med Hyg 20: 385-422.

38. Cerqueira NL (1950) Description of the larva, pupa, and female of *Aedes* (Finlaya) *argyrothorax* Bonne-Wepster and Bonne. Proc Ent Soc Wash 52: 173-178.

39. Morrison AC, Astete H, Chapilliquen F, Ramirez-Prada C, Diaz G, et al. (2004) Evaluation of a sampling methodology for rapid assessment of *Aedes aegypti* infestation levels in Iquitos, Peru. J Med Entomol 41: 502-510.

40. Morrison A, Gray K, Getisb A, Astete H, Sihuinchac M, et al. (2004) Temporal and geographic patterns of *Aedes aegypti* (Diptera: Culicidae) production in Iquitos, Peru. J Med Entomol 41: 1123-1142.

41. R Development Core Team (2008) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

42. Venables WN, Ripley BD (2002) Modern Applied Statistics with S. New York: Springer.

43. Ramasamy R, Surendran SN, Jude PJ, Dharshini S, Vinobaba M (2011) Larval development of *Aedes aegypti* and *Aedes albopictus* in peri-urban brackish water and its implications for transmission of arboviral diseases. PLoS Negl Trop Dis 5: e1369.

44. Wong J, Morrison AC, Stoddard ST, Astete H, Chu YY, et al. (2012) Linking oviposition site choice to offspring fitness in *Aedes aegypti*: consequences for targeted larval control of dengue vectors. PLoS Negl Trop Dis 6: e1632.

45. Garelli FM, Espinosa MO, Weinberg D, Trinelli MA, Gurtler RE (2011) Water use practices limit the effectiveness of a temephos-based *Aedes aegypti* larval control program in Northern Argentina. PLoS Negl Trop Dis 5: e991.

46. Rabaa MA, Simmons CP, Fox A, Le MQ, Nguyen TTT, et al. (2013) Dengue Virus in Sub-tropical Northern and Central Viet Nam: Population Immunity and Climate Shape Patterns of Viral Invasion and Maintenance. PLoS Negl Trop Dis 7: e2581.

47. Rabaa MA, Ty Hang VT, Wills B, Farrar J, Simmons CP, et al. (2010) Phylogeography of recently emerged DENV-2 in southern Viet Nam. PLoS Negl Trop Dis 4: e766.

48. Hastings A, Cuddington K, Davies KF, Dugaw CJ, Elmendorf S, et al. (2005) The spatial spread of invasions: new developments in theory and evidence. Ecol Lett 8: 91-101.

49. Xia Y, Bjornstad ON, Grenfell B (2004) Measles metapopulation dynamics. Amer Nat 164: 267-281.

50. Bossenbroek JM, Kraft CE, Nekola JC (2001) Prediction of long-distance dispersal using gravity models: Zebra mussel invasion of inland lakes. Ecol App 11: 1778-1788.

51. Huber K, Loan LL, Chantha N, Failloux AB (2004) Human transportation influences *Aedes aegypti* gene flow in Southeast Asia. Acta Trop 90: 23-29.

52. Goncalves da Silva A, Cunha IC, Santos WS, Luz SL, Ribolla PE, et al. (2012) Gene flow networks among American *Aedes aegypti* populations. Evol Appl 5: 664-676.

53. Hemme RR, Thomas CL, Chadee DD, Severson DW (2010) Influence of urban landscapes on population dynamics in a short-distance migrant

mosquito: evidence for the dengue vector *Aedes aegypti*. PLoS Negl Trop Dis 4: e634.

54. Fulmali PVW, A.; Mahadev, P.V. (2008) Spread, establishment and prevalence of dengue vector *Aedes aegypti* (L.) in Konkan region, Maharashtra, India. Indian J Med Res 127: 589-601.
55. Gubler DJ (2011) Dengue, Urbanization and Globalization: The Unholy Trinity of the 21(st) Century. Trop Med Health 39: 3-11.
56. Kuno G (1997) Factors influencing the transmission of dengue viruses. In: Gubler DJ, Kuno G, editors. Dengue and dengue hemorrhagic fever. Cambridge, UK: CAB International. pp. 61-88.
57. Mahabir RS, Severson DW, Chadee DD (2012) Impact of road networks on the distribution of dengue fever cases in Trinidad, West Indies. Acta Trop 123: 178-183.
58. Kuno G (1995) Review of the factors modulating dengue transmission. Epidemiol Rev 17: 321-335.
59. Fouque F, Reynes JM, Moreau JP (1995) Dengue in French Guiana, 1965-1993. Bull Pan Am Health Organ 29: 147-155.
60. Focks DA, Daniels E, Haile DG, Keesling JE (1995) A simulation model of the epidemiology of urban dengue fever: literature analysis, model development, preliminary validation, and samples of simulation results. Am J Trop Med Hyg 53: 489-506.

61. Schmidt WP, Suzuki M, Thiem VD, White RG, Tsuzuki A, et al. (2011) Population density, water supply, and the risk of dengue fever in Vietnam: cohort study and spatial analysis. PLoS Med 8: e1001082.

62. Reiter P, Sprenger D (1987) The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes. J Am Mosq Control Assoc 3: 494-501.

63. Bataille A, Cunningham AA, Cedeno V, Cruz M, Eastwood G, et al. (2009) Evidence for regular ongoing introductions of mosquito disease vectors into the Galapagos Islands. Proc Biol Sci 276: 3769-3775.

64. Foley EA, Khatchikian CE, Hwang J, Ancca-Juarez J, Borrini-Mayori K, et al. (2013) Population structure of the Chagas disease vector, Triatoma infestans, at the urban-rural interface. Mol Ecol 22: 5162-5171.

65. Lounibos LP (2002) Invasions by insect vectors of human disease. Annu Rev Entomol 47: 233-266.

Chapter 3: River Boats Contribute to the Regional Spread of Dengue Vector *Aedes aegypti* in the Peruvian Amazon

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3.1 Introduction

Anthropogenic changes such as increased trade, rapid transportation, and population movements favor the introduction and establishment of invasive mosquitoes and pathogens [1]. Recent reports of dengue in Key West, Florida [2], West Nile Virus in the United States [3], and Chikungunya in the Caribbean [4] demonstrate the potential for introduced mosquitoes and pathogens to cause serious outbreaks. Accordingly, an understanding of the biological and ecological factors that facilitate establishment of a vector species in a new location can provide timely information to develop and implement vector surveillance and control, and in particular to suppress the geographic expansion of vector-borne pathogens.

An invasion process can be summarized as a series of sequential steps including transport to a new region, release into the new environment, establishment, and spread [5]. Several factors determine the success of an invading organism, including the number and frequency of introduction events (propagule pressure), key life history traits, behavior of the invading species, and abiotic and biotic properties of the receiving ecosystem [5-8]. Dengue vector

Aedes aegypti displays several characteristics that contribute to its rapid and ongoing spread through human transport activities including; egg desiccation resistance, anthropophilic blood-feeding, and oviposition in artificial water-holding containers commonly found in and around the home such as vases, plastic buckets, water storage tanks, and discarded refuse and tires [9-11].

Ae. aegypti dispersal occurs in one of two ways: 1) Adult *Ae. aegypti* may fly when seeking human hosts or oviposition sites, and 2) *Ae. aegypti* eggs, larvae, pupae, or adults may be passively transported from one place to another via anthropogenic activities. It is generally accepted that *Ae. aegypti* flight range is limited to ~100m and often much less [12-14], and therefore human activities are responsible for mosquito dispersal over longer distances. Human-mediated dispersal is supported by evidence from population genetics studies [15-17], in addition to field studies documenting *Ae. aegypti* on airplanes, boats, and trains [9,18,19] . Indeed, *Ae. aegypti* most likely was transported from West Africa to the Americas via trade ships in the 15th-19th centuries [10,20].

Since the waning of a Pan American Health Organization yellow fever control program in the 1960-70s [21,22], *Ae. aegypti* has been expanding from urban to peri-urban and rural areas throughout the Americas, including the Peruvian Amazon [23,24]. Our previous research demonstrates different spatial patterns of *Ae. aegypti* infestation in communities accessible by roads vs. rivers. *Ae. aegypti* expansion follows the linear configuration of highway communities, whereas no clear pattern exists in riverine communities [23]. Although environmental differences between settlements (i.e. - abundance of wet containers) may contribute to the heterogeneous infestation pattern, it is also possible that varying degrees of frequency and intensity of new mosquito introductions (propagule pressure) contribute to the spatial pattern of *Ae. aegypti* geographical spread. That is, some towns are likely to have more frequent introductions than others.

Insect vector invasion via human transportation networks has been previously described, e.g., *Culex quinquifasciatus* transport via airplanes in the Galapagos [25], *Triatoma infestans* movement via human activities in Peru [26], and *Aedes albopictus* movement through tire trade [27]. Morrison et al (2006) showed *Ae. aegypti* infestation in Iquitos ports and in large barges, but did not characterize temporal trends of infestation or the extent of vehicle infestation patterns [18]. In the present study we specifically address propagule pressure through an analysis of *Ae. aegypti* infestation rates of different vehicle types in a relatively isolated region of the Peruvian Amazon. We compared *Ae. aegypti* adult and immature infestation levels of various vehicle type (boats, buses, taxis, etc.) and between periods of high and low precipitation. Our approach provides empirical data on vehicle infestation that can be combined with transportation data to provide more accurate estimates of propagule pressure, thus aiding in our ability to ultimately predict *Ae. aegypti* range expansion and mitigate future dengue outbreaks.

3.2 Methods

Ethics Statement

Permission for this study was granted by the Loreto Regional Health Department, and the study protocol was approved by the NAMRU-6 Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects (protocol number NAMRU6.2012.0039). Vehicle operators provided oral consent to the collection of mosquitoes, and no personal information was collected during entomological surveys. In accordance with the NAMRU-6 IRB-approved protocol, we provided vehicle operators with handouts documenting mosquito collection procedures in detail. Written consent was not appropriate for this study, since our project only involved the collection of mosquitoes and no personal information was collected. In addition, the Emory University Institutional Review Board determined that this study does not represent human subjects based research.

Study Area

Iquitos is the most populous city in the Peruvian Amazon, with approximately 400,000 inhabitants in the metropolitan area. Although river networks are the predominant mode of transit, a 95 km road connecting Iquitos to the smaller city of Nauta (population: 17,000), facilitates terrestrial commerce and population movement. Seasonal fluctuations in Amazon River levels influence the degree of transit within the Peruvian Amazon: river transit is most intense when the river levels are intermediate (September-January, April-June) and less frequent during periods of extreme high or low river levels (highest in ~March, lowest in ~August) [28] .

Entomological Sampling

In February, May, August, and October of 2013 we surveyed different vehicle types for *Ae. aegypti* adult and immature mosquitoes. Weather in Iquitos exhibits seasonality, but the magnitude of change in temperature and precipitation is small [28]. Although precipitation occurs throughout the year, it is usually lowest between May and September (**S3.1 Fig**). Thus, we carried out two collections during periods when precipitation was high (February and October) and two collections during periods when precipitation and temperatures were lowest (May and August). The high precipitation period coincides with the dengue season in Iquitos, occurring from September to April [28].

Six different aquatic vehicle types and two different terrestrial vehicle types were surveyed. Aquatic vehicles included large barges (locally known as *lanchas*), medium-sized barges (*lanchitas*), speed boats (*rápidos*), and small water taxis (*peque-peques*) (**Fig 3.1**). Large barges (length ~30m) carry passengers and cargo throughout the Peruvian Amazon and have 2-3 floors including cargo holds in the bottom of the boat. Medium-sized barges (length ~20m) carry cargo and passengers locally in the Iquitos region and have 1-2 floors, but no cargo holds. Terrestrial vehicles included van-sized buses (*combis*) and taxis that travel along the Iquitos-Nauta highway (**Fig 3.2**) , the only major road out of Iquitos, a city which is only accessible by airplane or river travel (minimum of 3 days). For each sampling period, we surveyed between 7 and 17 vehicles of each type for adult and larval mosquitoes.

We surveyed boats and taxis/ buses at five different ports and three different bus stations/ taxi departure points in Iquitos. Vehicles were selected on the basis of departure times (only vehicles staying in port less than two days were included) and the willingness of the owners to participate in the study. We recorded the vehicle name and registration number (when applicable) to uniquely identify each vehicle.

Adult mosquitoes were captured using Prokopack aspirators [29] along the walls of each vehicle, and on every floor including cargo holds. Collection effort was proportional to vehicle size. All adult mosquitoes were transported to the lab, killed by freezing at -20ºC, and identified to species using taxonomic keys. When identification to species was not possible due to damage or descaling, mosquitoes were identified and tallied by genus. Male and female mosquitoes were separated and counted, and physiological stage for females was evaluated according to three categories: empty, partially engorged, or completely engorged. When applicable, we also recorded the locations of the mosquito collections within the vehicles (floors 1-3 or cargo holds).

We searched all vehicles for immature mosquitoes, although they were only found on large and medium barges. We thoroughly searched every floor of barges including cargo holds for immature mosquitoes in water-holding (wet) containers. In accordance with previously established protocols [11,30] , for each wet container we recorded the location by floor and room type (i.e.- cargo hold, kitchen, etc.), degree of organic material in the water (on a scale of 1 to 3, with 3 representing high concentrations of organic material), container type, solar exposure, fill method (active collection of water via human activities vs. passive, unintentional accumulation of water), presence of abate, and the presence of other mosquito species. All immature mosquitoes were collected in Whirl-pack bags (Nasco, Fort Atkinson, WI) and transported to the laboratory for rearing and identification to species using taxonomic keys. Immature mosquitoes were counted and tallied by species and life stage (egg, larvae, or pupae). After collecting immature mosquitoes, we either emptied the wet containers or we treated the container with Abate (temephos) larvicide to prevent further proliferation of mosquito populations.

Transportation Data

Simultaneous with entomological surveys, we interviewed vehicle drivers to determine the frequency of travel between Iquitos and surrounding towns for each vehicle type (and thus we were able to estimate the number of vehicles traveling to each town). Additionally, the final destinations for each vehicle were mapped using ArcGIS, and the path distances between final destinations and Iquitos were measured. We then calculated the average distance traveled for each vehicle type infested with *Ae. aegypti* mosquitoes.

Data Management and Analysis

Due to unpredictable vehicle transit patterns in Iquitos, on a number of occasions we sampled the same barge more than once. In order to adhere to statistical assumptions of independence we randomly selected which observation would be included in the dataset for all statistical tests. We assigned a unique identifier to each individual vehicle, and all vehicles that were only sampled on one occasion were included in the final subset of data. For vehicles that were sampled on multiple occasions, we assigned an additional identifier representing the sampling occasion. (For example a vehicle surveyed in both February and May would be assigned a sampling occasion numbers 1 and 2, respectively.) The sampling instance to be included in the subset of data was randomly selected using a random number generator. Subsampling left us with a total of 32 independent collections from large barges, 33 medium barges, 41 speed boats, 53 water taxis, 40 buses, and 30 taxis.

Fisher's exact tests were used to determine whether there was variation in proportion of infested vehicles within and between sampling periods. Further analysis was conducted only for large and medium-sized barges, where the vast majority of *Ae. aegypti* and other mosquitoes were found.

For these large and medium barges, the number of *Ae. aegypti* adults (total, females, and blood-feds) and immatures (larvae and pupae) per barge was calculated by date. Entomological indices were calculated, included the Premise (vehicle) Index (positive vehicles/ number inspected *100), the Container Index (positive containers/ number inspected *100), and the Breteau Index (positive containers/ vehicles inspected*100) [18]. Pupal productivity was calculated by location within the boat (by floor) and by wet container type [11]. Differences in abundance by location within medium barges were not tested, due to overall low *Ae. aegypti* abundance. Fisher's exact tests were used to compare the proportion of infested vs. uninfested containers by container type.

Nonparametric Kruskal-Wallis tests for median comparisons among two or more groups were used to test the null hypothesis of no significant differences in *Ae. aegypti* abundance (for either adult or immature mosquitoes) by date or by location within boats (floors 1-3, cargo holds). Abundance comparisons between the high precipitation (February, October) and low precipitation (May, August) dates were made using non-paired Mann-Whitney Wilcoxon tests. We also used non-paired Mann-Whitney Wilcoxon tests to determine whether adult and immature *Ae. aegypti* abundance was greater for Puerto Masusa (Iquitos' largest port) in comparison with all other sampling locations.

All data analysis was conducted using R Statistical Software [31].

3.3 Results

For all dates, large barges were the most heavily infested with *Ae. aegypti*, with an overall infestation rate of 71.9%, followed by medium barges (39.4% infested) and buses (12.5% infested) (**Fig 3.3**).No *Ae. aegypti* mosquitoes were found on taxis, water taxis, or speed boats. Differences in the proportion of vehicles infested were statistically significant within all months except for May

(Fisher's exact test $p < 0.003$ in all other cases). Since the majority of mosquitoes were found on large and medium-sized barges, the remainder of our analysis is focused exclusively on those vehicle types. **S3.1- S3.5 Tables** show a complete list of all mosquito species found by vehicle type.

Large barges traveled extensively throughout the Peruvian Amazon, with an average path distance of 570 km per trip, conducting 3.2 trips per month. Medium barges traveled an approximate path distance averaging 195 km from Iquitos, with 6.5 trips per month (approximately 1/3 of the total distance of large barges per trip, with twice as many trips each month). Buses traveled more locally but with much greater frequency, with a path distance of ~76 km, and an average of 51 trips per month.

Large Barges

A total of 9 species of mosquitoes from six different genera (all of which have been previously identified in the Iquitos region) were found on large barges (**S3.1 Table**) [32,33]. Of the mosquitoes identified to species (N=3850), *Culex quinquefaciastus* was the most common (62.6%), followed by *Ae. aegypti* (-29.8%), and *Culex coronator* (2.5%) (**Table 3.1**, **S3.1 Table**). *Cx. quinquefaciastus* and *Ae. aegypti* remained the two dominant mosquito species throughout all sampling dates. Of the 32 individual barges sampled, adult *Ae. aegypti* were found in 23 barges, and immature *Ae. aegypti* were found in 7 large barges. Among large barges that were positive for immature *Ae. aegypti*, 6 out of 7 also contained adults.

Ae. aegypti adult abundance was highly aggregated – approximately 25% of large barges (N=8) were responsible for 77.8% of all *Ae. aegypti* adults found. Adult *Ae. aeygpti* abundance did not differ significantly by date or between high and low precipitation periods. Approximately 75% of female *Ae. aegypti* collected (N=66) were blood-fed (**Table 3.2**). Within boats, cargo holds had a higher average number of adult *Ae. aegypti* than floors 1-3 (Kruskal-Wallis X^2= 9.80, $p< 0.05$) (**Fig 3.4**). This pattern was consistent across all dates, except for August when overall abundance was low and slightly more mosquitoes were found on the first floor (1.6 mosquitoes/ boat on the first floor vs. 1 mosquito/ boat in the cargo holds). Adult *Ae. aegypti* were more likely to be found in large barges in Iquitos' busiest port, Puerto Masusa, in comparison with all other ports sampled (Mann-Whitney Wilcoxon U=182.5, $p<0.05$).

Cx. quinquefasciatus and *Ae. aegypti* were the only two species of immature mosquitoes found in large barges. No differences in the numbers of *Ae. aegypti* pupae or larvae were detected by sampling date or by low vs. high precipitation months (**Table 3.3**). The proportion of positive containers was highest in October (Container Index: 9.8%), and the number of positive containers per 100 vehicles (Breteau Index: 85.7) was highest in August.

Interestingly, the proportion of vehicles positive for larvae or pupae was higher during sample dates with less precipitation, 30.8% for the May-August collections and 15.8% for the October-February collections. As with adult mosquitoes, the distribution of immature *Ae. aegypti* among barges was highly aggregated: 6.3% of barges (N=2) produced 93.6% of larvae and 76.7% of pupae.

On average we found 16.7 wet containers per barge across all dates (SD= 10.3), with no difference between the low and high precipitation periods (17.9 containers/ vehicle SD= 12.8 vs 17.5 containers/ vehicle, SD= 9.0; U=348, p>0.05). As with adult mosquitoes, immature *Ae. aegypti* were most likely to be found in cargo holds, accounting for 89.3% (N= 25) of positive habitats (X^2= 9.8, p<0.05) . The remaining positive habitats were found on the first (7.1%, N= 2) and second floors (3.6%, N= 1) of barges. Difference in the number of larvae and pupae by floor approached significance, with a greater number of immature mosquitoes found in the cargo holds (larvae: X^2=7.5, p= 0.06, pupae: X^2=7.8, p= 0.05) (**Fig 3.4**).

The preferred habitats of larvae (85.9%) and pupae (76.7%) were puddles formed on the boat floor in cargo holds. Other containers produced immature mosquitoes, including tires (10.4% of larvae and 3.5% of pupae) and dishes (3.7% of larvae and 19.8% of pupae). The proportion of *Ae. aegypti* positive floor puddles, tires, and dishes was significantly greater in comparison with other container types (Fisher's exact test p<0.05), but puddles in cargo holds were by far the most abundant habitat overall (N=396). Other containers were relatively rare (**Table 3.4**). Despite thorough inspection, no mosquito eggs were found on barges, likely due to the extremely dark conditions in cargo holds.

Of the 14 barges that were sampled more than once, 13 were positive for *Ae. aegypti* immatures or adults during at least one sampling occasion. In general, barges that were initially infested tended to remain infested over time: only two boats were initially infested and later found to be uninfested.

Medium Barges

On medium barges, 15 species of adult mosquitoes were found, with *Cx. quinquefasciatus* comprising 84.9% of all identified mosquitoes (**Table 3.1**, **S3.2 Table**). *Ae. aegypti* comprised 6.3% of mosquitoes, although overall abundance was significantly lower than for large barges (**Table 3.2**, N=23 *Ae. aegypti* adults found among independent observations, U= 787.5, p<0.0001). There were no significant differences in adult *Ae. aegypti* abundance by date or by high vs. low precipitation period (X^2= 4.0, p> 0.05; U=169, p>0.05, respectively).

Immature mosquitoes found on medium barges included *Ae. aegypti* and *Cx. declarator-mollis.* In comparison with large barges, immature indices on medium-sized barges were notably lower (**Table 3.3**). The proportion of boats positive for *Ae. aegypti* immatures was highest in August (Premise Index: 16.7), followed by February (Premise Index: 9.1). No immature mosquitoes were found on medium barges In May or in October. For all dates, about 0.9% of containers were positive (**Table 3.4**), and the types of containers infested differed significantly (tires, trash, and other container types, Fisher's exact test p< 0.01).

In contrast to large barges, *Ae. aegypti* infestation status on medium barges was less consistent. Only three medium barges that were initially infested with *Ae. aegypti* remained infested in subsequent dates.

3.4 Discussion

Few studies to date have actively monitored human transport vehicles for invasive species [19,25,34]. While *Ae. aegypti* has been previously documented on vehicles in Iquitos [18], we compared infestation across multiple vehicle types and across a full year capturing seasonal variation. We conclude that river boats are the most significant source *Ae. aegypti* regional spread in the Peruvian Amazon because 1) large and medium barges are frequently and heavily infested with mosquitoes, 2) the majority of towns in the Iquitos region are connected only by rivers, and 3) the spatial pattern of *Ae. aegypti* establishment in the region suggests a primarily riverine mode of spread [23]. Although buses traveled most frequently, their overall infestation rates were very low (no more than one mosquito per sampling event), and they traveled for much shorter distances

Ae. aegypti *and Dengue Invasion*

Our results support the hypothesis that in the Peruvian Amazon aquatic transit is most important for the spread of *Ae. aegypti* . Although buses had a lower infestation rate in comparison with barges, terrestrial routes are important for trade and transportation in most of the rest of the world. Further study is needed to determine whether vehicle infestation rates are similar in other areas, and the degree to which terrestrial traffic contributes to *Ae. aegypti* invasion.

Infestation rate, however, is only one component of propagule pressure, and a better measure of invasion risk would be the arrival of infested vehicles to new locations, as pointed out by Caton et al (2006). Infestation rate alone, therefore, may overestimate invasion risk, particularly when vehicle traffic is low, few mosquitoes are adults, or the male-female ratio is uneven [34].

Because there are no major highways in the Peruvian Amazon, regional transportation is predominantly aquatic: large barges frequently carry up to ~200 passengers to Iquitos from major population centers such as Pucallpa (approximately 200,000 inhabitants). It is therefore probable that infected individuals introduce dengue viruses to Iquitos by boat. Heavy mosquito infestation on boats could also lead to incipient virus transmission during travel. Trips can last several days (and in some cases weeks), leaving ample time for mosquitoes to take several blood meals. Stoddard et al (2014) described Amazon River levels as increasing during the first and third trimesters of the year (September-March), which are also periods of high dengue transmission. Since river traffic appears to be more intense during periods of intermediate river levels, we would expect more mosquito and dengue introductions during that time.

Dengue control strategies are very limited and focus on the reduction of mosquito infestations in urban areas where dengue cases have been detected: only rarely is control done preemptively. Results from this study, in conjunction with our previous findings [23], imply that *Ae. aegypti* mosquitoes move between Iquitos and surrounding towns. Population genetics studies could be used to characterize metapopulation structure of *Ae. aegypti* in the Iquitos region, and identify which routes (terrestrial or aquatic) are most relevant for gene flow. Evidence for fluid migration between subpopulations would indicate that rural settlements may serve as refuge sources for reinfestation or, given the limited vector control in such towns, potential sources of mosquitoes that are susceptible to insecticides.

Knowledge Gaps about* Ae. aegypti *Infestation of Boats

It is not surprising that in the Peruvian Amazon *Ae. aegypti* colonization of barges is extremely common, given that these vehicles provide all of the mosquito's lifecycle needs: a captive group of human hosts for blood meals, abundant oviposition sites, and dark, cool resting places for adults. There are two means by which mosquitoes might initially colonize a boat: flight of adults in search of oviposition sites [14] or humans unintentionally carrying infested containers aboard. It is unclear however, which of these mechanisms is most common and how seasonality might influence the colonization process. Mark-release-recapture experiments in empty port areas could be employed to compare the relative "attractiveness" of boats vs. houses in different seasons. Human-mediated dispersal could be measured through intensive longitudinal monitoring of artificial containers being loaded into vehicles. Of course, both mark-release-recapture experiments and longitudinal monitoring of artificial containers on boats would be labor-intensive. Indeed, despite inspecting 971 different containers on 34 different large barges, we were unable to locate a single mosquito egg.

The most important seasonal change observed throughout the study was that collections from August (lower precipitation) showed a greater proportion of boats infested with immature *Ae. aegypti* (despite overall lower mosquito abundance). These patterns may be a result of *Ae. aegypti* searching more expansively for oviposition sites, due to scarcity of rain-filled wet containers in port areas during the periods of low precipitation [14]. Indeed, the number of wet containers on large barges did not differ between dates, possibly because the most common immature habitats (ground puddles in cargo holds) are primarily filled through cleaning activities and possibly through rainfall (**Fig 3.5**).

Management of* Ae. aegypti *as an Invasive Species

Our findings indicate that periodic administration of larvicides alone is insufficient for mosquito control on boats. Despite administering temephos, we often found the same vehicle infested again some months later. Therefore, we propose an integrated approach that combines larvicide administration with habitat source reduction through improved boat construction, maintenance, and cleaning. Adulticides should also be employed during periods of high mosquito abundance. Though local law in Iquitos mandates that boats be sprayed periodically with insecticides, this is rarely done since the laws are not enforced and boat owners have no economic incentive to carry out mosquito control. Accordingly, we propose that governmental bodies invest in mosquito control activities through existing infrastructure for aquatic transit (such as port authorities). Both punitive policies (such as fines) and incentivizing policies (such as tax breaks) could be implemented to ensure individual cooperation with mosquito control activities. In some cases active surveillance and control of mosquito populations in airplanes and ports has been conducted to allow for early detection and rapid intervention of invasive species [19], although in resource-poor environments this unlikely to be a realistic solution. The aggregate distribution of adult and immature mosquitoes suggests that some boats may act as super-transporters of mosquitoes, just as individual hosts may act as super-spreaders of pathogens [35]. Taking this into account, vector control programs might target those vehicles producing the greatest amount of mosquitoes. In our collections, we observed that infested boats tended to be older, and were more likely to have rust holes that allow water to drip between floors and collect in cargo holds to form puddles.

3.5 Tables

Table 3.1. Most commonly found adult mosquitoes found on large and medium barges and buses. The most common mosquito species found included *Cx. quiquefasciatus*, *Cx. coronator*, and *Ae. aegypti*. (See **Tables S3-S7** for a complete list of mosquito species found by vehicle type.)

	Culex* (*Culex*) *quinquefasciatus			***Culex* (*Culex*) *coronator***			***Aedes* (*Stegomyia*) *aegypti***		
	Large Barges	**Med. Barges**	**Buses**	**Large Barges**	**Med. Barges**	**Buses**	**Large Barges**	**Med. Barges**	**Buses**
All Months	2409	1060	4	96	8	0	1110	79	7
February	697	9	1	95	8	0	89	10	3
May	510	579	2	1	0	0	144	7	3
August	219	65	0	0	0	0	52	9	0
October	983	407	1	0	0	0	825	53	1

Table 3.2. Ae. aegypti adult mosquitoes on large and medium barges. The data shown in the table below only includes independent instances of sampling for large (N= 32) and medium (N= 33) barges. Numbers in parenthesis refer to the proportion of adult mosquitoes, females, or blood-fed mosquitoes per barge.

	February		**May**		**August**	
	Large Barges	**Med. Barges**	**Large Barges**	**Med. Barges**	**Large Barges**	**Med. Barges**
No. Barges Sampled	11	11	6	9	7	6
No. Adults (Adults/ Barge)	58 (5.27)	8 (0.73)	14 (2.33)	1 (0.11)	22 (3.14)	6 (1)
No. Females (Females/ Barge)	35 (3.18)	3 (0.27)	5 (0.833)	0	16 (2.29)	4 (0.67)
No. Blood-feds (Blood-feds/ Barge)	17 (1.56)	1 (0.09)	4 (0.67)	0	15 (2.14)	4 (0.67)

Table 3.2 Continued

	October		All Months	
	Large Barges	**Med. Barges**	**Large Barges**	**Med. Barges**
No. Barges Sampled	8	7	**32**	33
No. Adults (Adults/ Barge)	50 (6.25)	8 (1.14)	**144 (4.50)**	**23 (0.70)**
No. Females (Females/ Barge)	31 (3.88)	7 (1)	**87 (2.72)**	**14 (0.42)**
No. Blood-feds (Blood-feds/ Barge)	30 (3.75)	6 (0.86)	**66 (2.06)**	**11 (0.33)**

Table 3.3. Immature indices by month for large and medium barges. The data shown in the table below only includes independent instances of sampling for large (N= 32) and medium (N= 33) barges. Container (positive containers/ number inspected *100), Breteau (positive containers/ premises inspected*100), and Premise Indices (positive premises/ number inspected *100), were calculated using the presence of either larvae or pupae. Entomological indices were adapted for vehicle surveillance so that an individual vehicle was counted as a 'premise' [18].

	February		**May**		**August**	
	Large Barges	**Med. Barges**	**Large Barges**	**Med. Barges**	**Large Barges**	**Med. Barges**
No. barges sampled	11	11	6	9	7	6
Positive Barges	1	1	0	0	4	1
Premise Index	9.09	9.09	0	0	57.14	16.67
Container Index	4.04	1.15	0	0	6.32	2.22
Breteau Index	72.73	9.09	0	0	85.71	16.67
***Ae. aegypti* larvae (larvae/ vehicle)**	551 (50.09)	8 (0.73)	0	0	33 (4.71)	20 (3.33)
***Ae. aegypti* pupae (pupae/ vehicle)**	49 (4.45)	0	0	0	20 (2.9)	0

Table 3.3 Continued

	October		All Months	
	Large Barges	**Med. Barges**	**Large Barges**	**Med. Barges**
No. barges sampled	8	7	**32**	**33**
Positive Barges	2	0	**7**	**2**
Premise Index	25	0	**21.88**	**6.06**
Container Index	9.79	0	**5.33**	**0.86**
Breteau Index	175	0	**87.5**	**6.06**
***Ae. aegypti* larvae (larvae/ vehicle)**	126 (15.75)	0	**710 (22.19)**	**28 (0.85)**
***Ae. aegypti* pupae (pupae/ vehicle)**	17 (2.13)	0	**86 (2.69)**	**0**

Table 3.4. Proportion of positive containers by type – large barges. A container was considered to be positive if it contained *Ae. aegypti* at any immature stage (eggs, larvae, or pupae). On large barges significant differences were found in terms of floor puddles, dishes, and tires, and other container types (Fisher's exact test $p<0.05$). On medium barges significant differences were detected between tires, trash, and other container types (Fisher's exact test $p<0.01$).

	Large Barges	**Medium Barges**
	No. positive/ No. inspected (%)	**No. positive/ No. inspected (%)**
Tires	2/ 23 (8.70)	1/ 46 (2.17)
Floor puddles	25/ 396 (6.31)	0/ 91
Dishes (plates, mugs, plate holders)	1/ 20 (5)	0/ 13
Other (tanks/ drums, plastic containers)	0/80	0/ 78
Trash (discarded items)	0/ 6	1/5 (20)
Total	**28/ 525 (5.33)**	**2/ 233 (0.86)**

3.6 Figures

Figure 3.1. Vehicle types surveyed. Aquatic vehicles surveyed included (clockwise from upper left); large barges (*lanchas*), medium-sized barges (*lanchitas*), small water taxis (*peque-peques*), and speed boats (*rápidos*). Terrestrial vehicles surveyed included buses (*combis*) and taxis.

Figure 3.2. Common transportation routes in the Iquitos region. Transportation is dominated by fluvial activity, with the exception of a 95km highway running from Iquitos to Nauta.

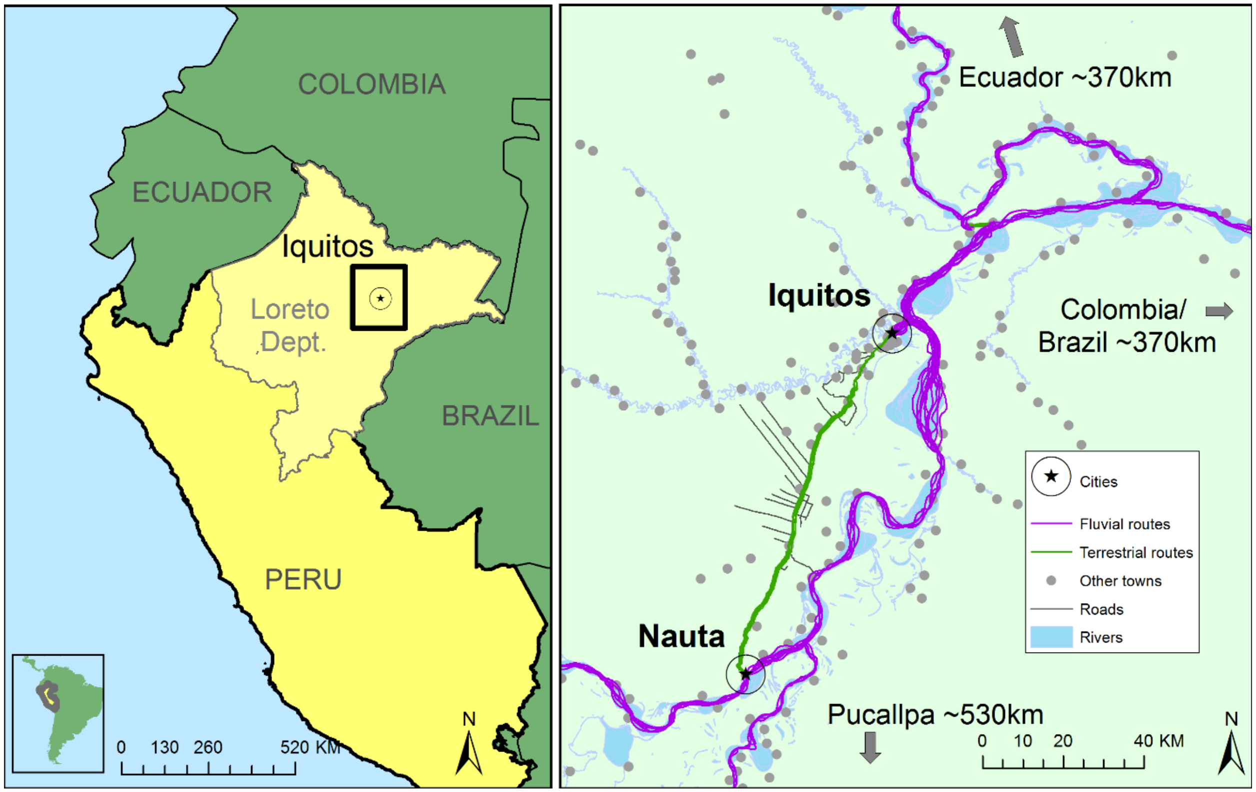


Figure 3.3. Proportion of vehicles infested with Ae. aegypti mosquitoes. Vehicles were considered to be infested with *Ae. aegypti* if either adult or immature mosquitoes were found. Bars show 95% confidence intervals for proportions. Note that some barges were sampled repeatedly across seasons (N=14), and within seasons (N=2).

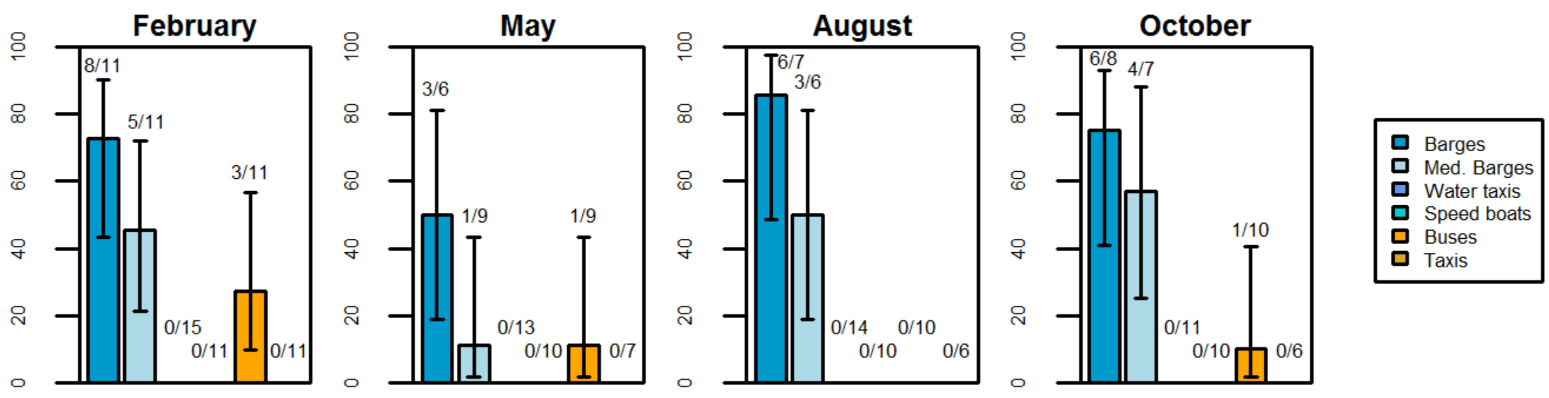


Figure 3.4. Ae. aegypti adults and immatures per boat by location for all periods - large barges. The vast majority of *Ae. aegypti* immature mosquitoes (A) and adult mosquitoes (B) were found in cargo holds.

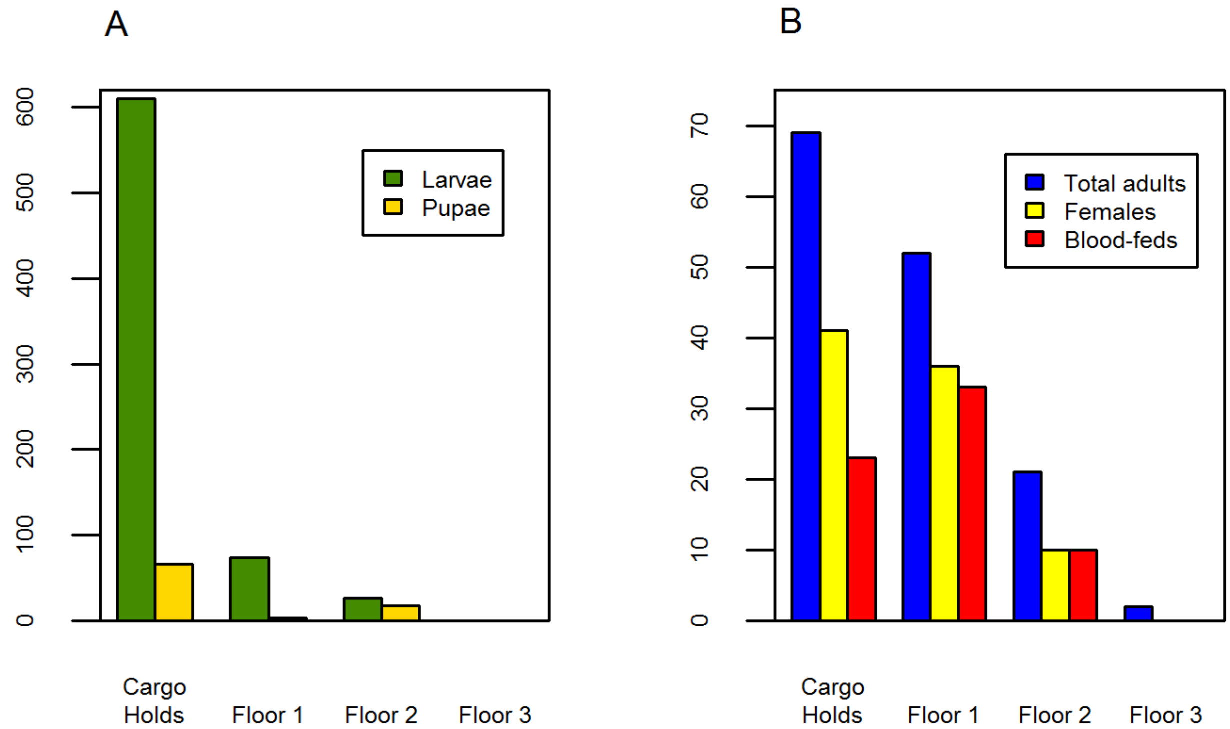
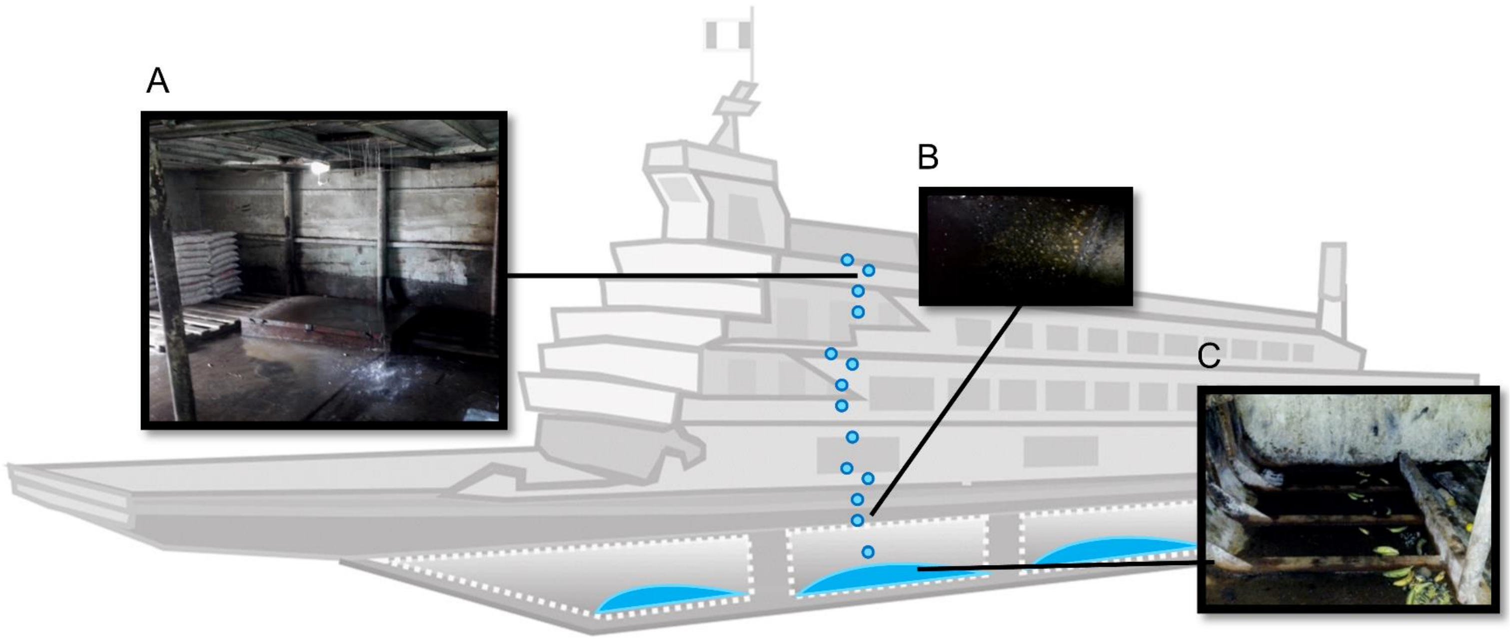


Figure 3.5. Formation of puddles in cargo holds. Barges are typically mopped whenever the boat is docked at either its origin or destination point. Water from rain and cleaning activities drips from upper most floors (A) through rust holes in the cargo hold roof (B). Water accumulates in the bottom of the cargo holds (C), and *Ae. aegypti* eggs are laid on the edges of the puddles, hatching when the puddles are refilled with water.



<u>3.7 Supplementary Tables</u>

Table S3.1. Adult mosquitoes found on large barges by season. In some cases mosquito samples were damaged and could only be identified to genus or subgenus (denoted by spp.).

Genus	**(Subgenus) species**	**All months**	**February**	**May**	**August**	**October**
Culex						
	spp.	3618	1299	657	352	1310
	(*Culex*) *quinquefasciatus*	2409	697	510	219	983
	(*Culex*) *coronator*	96	95	1	0	0
Aedes						
	(*Stegomyia*) *aegypti*	1110	89	144	52	825
Mansonia						
	(*Mansonia*) *titillans* or *indubitans*	13	2	5	5	1
	(*Mansonia*) *titillans*	33	5	7	10	11
	(*Mansonia*) *indubitans*	31	24	1	6	0
	(*Mansonia*) *humeralis*	15	2	5	7	1
	(*Mansonia*) *amazonensis*	1	0	1	0	0

Aedomyia						
	(*Aedomyia*) *squamipennis*	12	7	5	0	0
Coquillettidia						
	(Rhynchotaenia) *venezuelensis*	2	0	0	0	2
Limatus						
	spp.	1	1	0	0	0
	Total	**7469**	**2318**	**1354**	**654**	**3143**

Table S3.2. Adult mosquitoes found on medium barges by season. In some cases mosquito samples were damaged and could only be identified to genus or subgenus (denoted by spp.).

Genus	(Subgenus) species	All months	February	May	August	October
Culex						
	spp.	1372	20	695	140	517
	(*Culex*) *quinquefasciatus*	1060	9	579	65	407
	(*Culex*) *coronator*	8	8	0	0	**0**
	(Culex) declarator-mollis	30	5	25	0	0
	(*Phenacomyia*) corniger	2	1	1	0	0
	(*Melanoconion*) spp.	1	0	0	0	1
	(Melanoconion) adamesi	8	8	0	0	0
	(*Melanoconion*) *spissipes*	1	0	0	1	0
	(*Melanoconion*) *ocossa*	1	0	0	0	1
	(Aedinus) *amazonensis*	1	0	1	0	0
Aedes						

	(*Stegomyia*) *aegypti*	79	10	7	9	53
Mansonia						
	(*Mansonia*) *titillans* or *indubitans*	2	0	2	0	0
	(*Mansonia*) *titillans*	20	5	3	5	7
	(*Mansonia*) *indubitans*	19	1	10	8	0
	(*Mansonia*) *humeralis*	9	1	3	2	3
Aedomyia						
	(*Aedomyia*) *squamipennis*	4	1	3	0	0
Coquillettidia						
	spp.	1	0	1	0	0
	(Rhynchotaenia) *venezuelensis*	3	1	0	0	2
Ochlerotatus						
	(*Protoculex*) *serratus*	1	0	0	0	1
	Total	**2622**	**70**	**1330**	**230**	**992**

Table S3.3. Adult mosquitoes found on buses by season. In some cases mosquito samples were damaged and could only be identified to genus or subgenus (denoted by spp.).

Genus	**(Subgenus) species**	**All months**	**February**	**May**	**August**	**October**
Culex						
	spp.	8	6	2	0	0
	(*Culex*) spp.	3	0	0	3	0
	(*Culex*) *quinquefasciatus*	4	1	2	0	1
Aedes						
	(*Stegomyia*) *aegypti*	7	3	3	0	1
Anopheles						
	spp.	1	1	0	0	0
	Total	**23**	**11**	**7**	**3**	**2**

Table S3.4. Adult mosquitoes found on speed boats by season. In some cases mosquito samples were damaged and could only be identified to genus or subgenus (denoted by spp.).

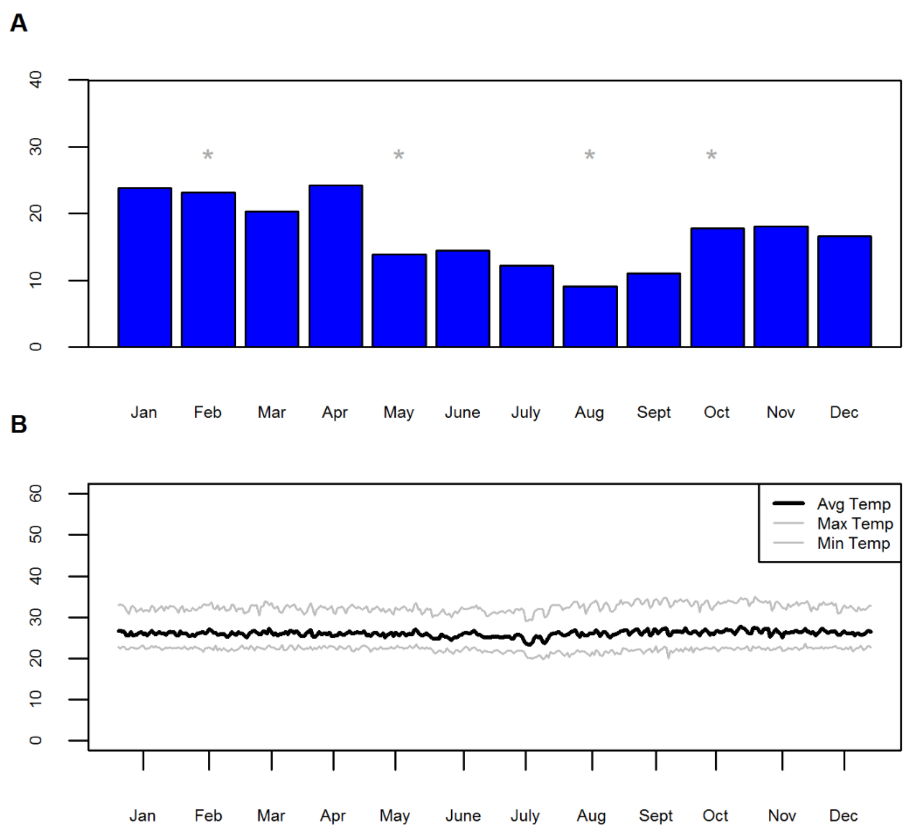
Genus	**(Subgenus) species**	**All months**	**February**	**May**	**August**	**October**
Culex		0	0	0	0	0
	(*Culex*) *quinquefasciatus*	5	0	5	0	0
	(Melanoconion) spp.	1	0	1	0	0
Mansonia		0	0	0	0	0
	(*Mansonia*) *psuedotitillans*	1	0	1	0	0
	(*Mansonia*) *titillans*	1	0	1	0	0
	Total	**8**	**0**	**8**	**0**	**0**

Table S3.5. Adult mosquitoes found on water taxis by season. In some cases mosquito samples were damaged and could only be identified to genus or subgenus (denoted by spp.).

Genus	**(Subgenus) species**	**All months**	**February**	**May**	**August**	**October**
Culex						
	(Melanoconion) spp.	1	0	1	0	0
	Total	**1**	**0**	**1**	**0**	**0**

3.8 Supplementary Figures

Figure S3.1. NOAA precipitation and rainfall data for Iquitos. A) Average monthly rainfall (cm) for 2009-2013 and B) Daily average, minimum, and maximum temperatures for 2009-2013. The symbol * on the graph indicates the months in which sampling took place.



3.9 References Cited

1. Alirol E, Getaz L, Stoll B, Chappuis F, Loutan L (2011) Urbanisation and infectious diseases in a globalised world. Lancet Infect Dis 11: 131-141.

2. Centers for Disease Control and Prevention (2010) Locally acquired Dengue--Key West, Florida, 2009-2010. MMWR Morb Mortal Wkly Rep 59: 577-581.

3. Reisen WK (2013) Ecology of West Nile virus in North America. Viruses 5: 2079-2105.

4. Centers for Disease Control and Prevention (2014) Notes from the field: chikungunya virus spreads in the Americas - Caribbean and South America, 2013-2014. Ann Emerg Med 64: 552-553.

5. Lockwood JL, Cassey P, Blackburn T (2005) The role of propagule pressure in explaining species invasions. Trends Ecol Evol 20: 223-228.

6. Williamson MH (1996) Biological Invasions. London; New York: Chapman & Hall.

7. Chapple DG, Simmonds SM, Wong BBM (2012) Can behavioral and personality traits influence the success of unintentional species introductions? Trends Ecol Evol 27: 57-64.

8. Levine JM, D'Antonio CM (1999) Elton revisited: a review of evidence linking diversity and invasibility. Oikos 87: 15-26.

9. Soper FL (1967) Dynamics of *Aedes aegypti* distribution and density. Seasonal fluctuations in the Americas. Bull World Health Organ 36: 536-538.

10. Christopher SR (1960) *Aedes aegypti* (L.), the yellow fever mosquito. Its life history, bionomics and structure. . Cambridge, UK: Cambridge University Press.

11. Morrison AC, Gray K, Getis A, Astete H, Sihuincha M, et al. (2004) Temporal and geographic patterns of *Aedes aegypti* (Diptera: Culicidae) production in Iquitos, Peru. J Med Entomol 41: 1123-1142.

12. Muir LE, Kay BH (1998) *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. Am J Trop Med Hyg 58: 277-282.

13. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg 72: 209-220.

14. Edman JD, Scott TW, Costero A, Morrison AC, Harrington LC, et al. (1998) *Aedes aegypti* (Diptera: Culicidae) movement influenced by availability of oviposition sites. J Med Entomol 35: 578-583.

15. Huber K, Loan LL, Chantha N, Failloux AB (2004) Human transportation influences *Aedes aegypti* gene flow in Southeast Asia. Acta Trop 90: 23-29.

16. da Silva AG, Cunha ICL, Santos WS, Luz SLB, Ribolla PEM, et al. (2012) Gene flow networks among American *Aedes aegypti* populations. Evolutionary Applications 5: 664-676.

17. Lima RS, Scarpassa VM (2009) Evidence of two lineages of the dengue vector *Aedes aegypti* in the Brazilian Amazon, based on mitochondrial DNA ND4 gene sequences. Genetics and Molecular Biology 32: 414-422.

18. Morrison AC SM, Stancil JD, Zamora E, Astete H, Olson JD, Vidal-Ore, Scott TW (2006) *Aedes aegypti* (Diptera: Culicidae) production from nonresidential sites in the Amazonian city of Iquitos, Peru. Annals of Tropical Medicine and Parasitology 100: S73–S86.

19. Sukehiro N, Kida N, Umezawa M, Murakami T, Arai N, et al. (2013) First report on invasion of yellow fever mosquito, *Aedes aegypti*, at Narita International Airport, Japan in August 2012. Jpn J Infect Dis 66: 189-194.

20. Carter HR (1931) Yellow fever. An epidemiologicaland historical study of its place of origin. Baltimore, Maryland: Williams & Wilkins Co. .

21. W SFWDLSA (1943) The organization of permanent nationwide anti-*Aedes aegypti* measures in Brazil. In: Foundation TR, editor. New York.

22. LB SDC (1974) A review of the status of yellos fever and *Aedes aegypti* eradication programs in the Americas. Mosquito News 34: 1-9.

23. Guagliardo SA, Barboza JL, Morrison AC, Astete H, Vazquez-Prokopec G, et al. (2014) Patterns of Geographic Expansion of *Aedes aegypti* in the Peruvian Amazon. PLoS Negl Trop Dis 8: e3033.

24. Diaz-Nieto LM, Macia A, Perotti MA, Beron CM (2013) Geographical limits of the Southeastern distribution of *Aedes aegypti* (Diptera, Culicidae) in Argentina. PLoS Negl Trop Dis 7: e1963.

25. Bataille A, Cunningham AA, Cedeno V, Cruz M, Eastwood G, et al. (2009) Evidence for regular ongoing introductions of mosquito disease vectors into the Galapagos Islands. Proc Biol Sci 276: 3769-3775.

26. Foley EA, Khatchikian CE, Hwang J, Ancca-Juarez J, Borrini-Mayori K, et al. (2013) Population structure of the Chagas disease vector, Triatoma infestans, at the urban-rural interface. Mol Ecol 22: 5162-5171.

27. Reiter P, Sprenger D (1987) The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes. J Am Mosq Control Assoc 3: 494-501.

28. Stoddard ST, Wearing HJ, Reiner RC, Jr., Morrison AC, Astete H, et al. (2014) Long-Term and Seasonal Dynamics of Dengue in Iquitos, Peru. PLoS Negl Trop Dis 8: e3003.

29. Vazquez-Prokopec GM, Galvin WA, Kelly R, Kitron U (2009) A new, cost-effective, battery-powered aspirator for adult mosquito collections. J Med Entomol 46: 1256-1259.

30. Morrison AC, Astete H, Chapilliquen F, Ramirez-Prada C, Diaz G, et al. (2004) Evaluation of a sampling methodology for rapid assessment of Aedes aegypti infestation levels in Iquitos, Peru. J Med Entomol 41: 502-510.

31. R Core Development Team (2008) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

32. Pecor JE, Jones J, Turell MJ, Fernandes R, Carbajal F, et al. (2000) Annotated checklist of the mosquito species encountered during arboviral studies in Iquitos, Peru (Diptera: Culicidae). J Am Mosq Control Assoc 16: 210-218.

33. Johnson MF, Gomez A, Pinedo-Vasquez M (2008) Land use and mosquito diversity in the Peruvian Amazon. J Med Entomol 45: 1023-1030.

34. Caton BP, Dobbs TT; Brodel CF (2006) Arrivals of hitchhiking insect pests on international cargo aircraft at Miami International Airport. Biological Invasions 8: 765-785.

35. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM (2005) Superspreading and the effect of individual variation on disease emergence. Nature 438: 355-359.

Chapter 4: Evidence for *Aedes aegypti* Oviposition on Boats in the Peruvian Amazon

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4.1 Introduction

Dengue vector *Aedes aegypti* is an invasive mosquito - originally West African in origin, it is widely accepted that *Ae. aegypti* was transported to the Americas via trade ships in the 17^{th}-19^{th} centuries [1]. Following the waning of a Pan American Health Organization yellow fever control program in the mid-1900s, *Ae. aegypti* began re-invading urban centers throughout Latin America [2]. Recent reports from Argentina and Peru exemplify *Ae. aegypti* geographic expansion from urban to peri-urban and rural areas [3,4].

Ae. aegypti mosquitoes are highly adapted to human environments: females feed almost exclusively on humans, prefer to rest in dark, cool areas (usually indoors) [5], and adult female mosquitoes lay their eggs on the inner walls of water-filled artificial containers found in and around the home such as vases, plastic buckets, bird baths, water storage tanks, and discarded refuse and tires. The potential of these containers to support immature *Ae. aegypti* is dependent on biotic factors such as bacteria, fungi, algae, as well as abiotic factors including pH, temperature, dissolved solids, and dissolved oxygen. A wide variety of microbes have been identified in *Ae. aegypti* breeding containers [6]. These microbes play important roles in larval nutrition as well as attraction and oviposition stimulation of gravid female mosquitoes [7-9]. This mosquito's adaptation to human environments, coupled with the longevity and resistance of its eggs to desiccation [10,11], contribute to the vector's passive spread to new areas via human transportation networks [12].

Our previous research demonstrated infestation of immature and adult *Ae. aegypti* on large barges in and around the Amazonian city of Iquitos, Peru [13] [14]. Results from these studies showed that Amazonian barges provide ideal conditions for all stages of the mosquito life cycle: there are abundant oviposition sites (in the form of floor puddles in cargo holds), ample human hosts for blood meals, and dark, humid resting sites for adult mosquitoes. Despite persistent *Ae. aegypti* infestation of boats, several questions about the infestation process remain (discussed in further detail, [13]). In this Short Communication, we address the question: Does *Ae. aegypti* oviposition occur during boat travel? The mere presence of immature mosquitoes on barges does not offer any information about the timing of oviposition. In other words, two possibilities exist: 1) Port populations of gravid females may fly aboard docked barges in search of oviposition sites and/ or 2) *Ae. aegypti* females on barges may oviposit during transit. We tested the hypothesis that *Ae. aegypti* oviposition occurs during boat travel by setting baited ovitraps on barges prior to departure from Iquitos, and collecting and examining the traps upon return to Iquitos.

Findings from this study have implications for the control of *Ae. aegypti* spread. If oviposition occurs while boats are docked, then vector control authorities could effectively apply larvicides and adulticides while boats are docked in the city of Iquitos. However, if *Ae. aegypti* oviposition occurs during boat travel, this implies that mosquito populations colonize and thrive on boats, thus presenting more challenges to mosquito population control.

To our knowledge, this is the first study that has employed traps to monitor *Ae. aegypti* populations aboard vehicles. Such an approach may represent an innovative and cost-effective means for monitoring mosquito populations on vehicles.

4.2 Materials and Methods

Large barges were monitored for *Ae. aegypti* oviposition during the months of August 2013, October 2013, and February 2014, with 20 barges sampled during each month. Barges were selected for the study on the basis of owners' willingness to participate and departure time (only barges departing within 24 hours of ovitrap deployment were included). Congruent with previous protocols, ovitraps were red plastic cups filled ¾ water (volume = 56.5 in^3) and lined with paper [15]. Ovitraps were baited with a mixture of bio-active bacterial attractant (composed of four species of bacteria, Ponnusamy et al. in review) in calcium alginate beads and spinosad larvicide (Wesson et al. in review). The composition of the mixture in each ovitrap was 100mg of attractant and 240mg of spinosad granules (Natular™ G, Clarke®, Roselle, IL, USA). Six ovitraps were placed within each barge in a dark, secluded area to maximize the probability of oviposition.

The exact dates of trap deployment and collection were noted for each barge, and the total travel time was calculated. The number of days between ovitrap deployment and collection varied due to different travel routes and destinations. Therefore, an increasing duration of ovitrap deployment could result in greater probability of oviposition due to the accumulation of organic material in the water over time. To account for this, we used a t-test to compare the mean travel time in days for positive vs. negative boats.

Sterile bags (Whirlpak Co.) were used to transport immature mosquitoes to the field laboratory for taxonomic identification to species, and ovitrap paper was thoroughly inspected under a microscope for the presence of eggs. The number of *Ae. aegypti* eggs was tallied by individual ovitrap and boat, and the proportions of positive traps were calculated by boat and by month. A trap was considered positive if it contained at least one *Ae. aegypti* egg, larva, or pupa. In some cases ovitraps were knocked over or disappeared. To adjust for missing ovitraps we calculated the proportion positive using a denominator that only included the number of intact traps at the time of collection.

All data analysis and graphs were produced using R statistical software [16]. The Premise Index (positive vehicles/ number sampled *100) was calculated by date, and Fisher's exact tests were used to determine whether there were significant differences in the proportion of barges positive for *Ae. aegypti* by month. To measure abundance, we calculated the mean number of eggs, larvae, and pupae per trap by month.

4.3 Results

Approximately 75% of ovitraps that were set (271 traps of 360) were successfully recovered. The remaining 89 traps were either knocked over during transit or disappeared. The average trip duration (and therefore number of days between ovitrap deployment and collection) was 15.5 days (SD= 6.1), although trip duration did not differ for positive vs. negative boats ($t = 1.55$, $p > 0.1$).

Among positive ovitraps, the overwhelming majority of mosquitoes found were *Ae. aegypti*. Two *Culex quinquefasciatus* larvae were found together with four *Ae. aegypti* larvae in a single trap in February, but no *Culex* egg rafts were found at any time during the study. Immature *Ae. aegypti* mosquitoes were found in 22 individual ovitraps from 15 out of 60 barges (Premise Index 25%) across all sampling dates (**Table 4.1**). Over the course of the study period, three ovitraps were found with *Ae. aegypti* larvae but no eggs. (One of these traps contained both *Ae. aegypti* larvae and pupae in the absence of eggs.) One ovitrap contained both eggs and larvae, and the remaining traps contained *Ae. aegypti* eggs but no larvae or pupae. The proportion of positive boats was highest in the month of May (35%) followed by August and October (20% each), although these observed differences were not statistically significant (Fisher's exact test $p > 0.5$).

The distribution of *Ae. aegypti* egg abundance was highly aggregated: 2.6% of traps (N=7) were responsible for 71.8% of eggs found, and 1.5% of traps (N=4) were responsible for all (100%) of the larvae found. Similarly, 5% of boats were responsible for the 71.47% of eggs. The greatest abundance of eggs was found during the month of October (N=325, 4.1 eggs per collected trap), while the least number of eggs were collected in August (N=86, 0.9 eggs per trap) (**Table 4.2**). Larvae and pupae were only found during the month of February.

4.4 Discussion

Our results provide strong evidence that *Ae. aegypti* oviposition occurs during barge travel throughout the year. It is probable that barges can support entire *Ae. aegypti* populations: barges contain dark, secluded areas, abundant oviposition sites, and ample human hosts for blood meals. Although oviposition occurs during barge travel, this does not eliminate the possibility that oviposition may also occur when port populations of mosquitoes invade boats in search of oviposition sites. Regardless, we conclude that the source of *Ae. aegypti* oviposition on boats is gravid females within boats (rather than port-dwelling mosquitoes). Our previous research has shown that large Amazonian barges are consistently and commonly infested (71%) with *Ae. aegypti* adult mosquitoes [13]. Because this mosquito vector has a relatively short flight range (< 100m, and usually much less), we would expect that gravid females already inside barges are more likely to oviposit in the boat in comparison with port-dwelling mosquitoes. Further, relatively few towns in the Iquitos region are infested with *Ae. aegypti* mosquitoes [15]. Because large human population size increases the risk of *Ae. aegypti* establishment, and most settlements in the Peruvian Amazon are small (with populations <1000 people), it is highly unlikely that *Ae. aegypti* would be found in the majority of sites visited.

Our previous research has shown that ground puddles formed in the bottom of cargo holds serve as productive and common *Ae. aegypti* immature habitats. Therefore, it is likely that our estimates of oviposition frequency are conservative, as our ovitraps compete with “natural” habitats in the cargo holds. Even so, in comparison with other collection methods such as aspiration of adults, ovitraps represent a cost-effective and easy way to monitor mosquito populations. Local health authorities and vector control programs could use this approach to monitor the presence of *Ae. aegypti* on vehicles, and ultimately use this information to slow the spread of this invasive vector.

To complement our findings that a few boats are responsible for most mosquito production and movement over long distances, we now add the observation that a few oviposition sites are responsible for much of the larval production. Such information is relevant to the targeted control of larval habitats within boats.

4.5 Tables

Table 4.1. Proportion of positive boats by date. There were no significant differences in the proportion of boats with positive ovitraps by month (Fisher's exact test $p > 0.5$). The Premise Index describes the number of positive vehicles/ number sampled *100 [14].

Month	Number of Positive Boats	Number Sampled	Premise Index
August 2013	4	20	20%
October 2013	4	20	20%
February 2014	7	20	35%
Overall	15	60	25%

Table 4.2. Number of Ae. aegypti eggs and larvae per trap by month. The abundance of eggs and larvae found did not differ by month.

Month	Mean eggs per trap (N)	Mean larvae per trap (N)	Mean pupae per trap (N)	Collected Traps
August 2013	0.91 (86)	0	0	95
October 2013	4.06 (325)	0	0	80
February 2014	2.07 (199)	0.10 (10)	0.03 (3)	96
Overall	2.25 (610)	0.04 (10)	0.01 (3)	271

4.6 References Cited

1. Gubler DJ (1997) Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. Dengue and dengue hemorrhagic fever. Cambridge, UK CAB International pp. 1-22.

2. (1986) *Aedes aegypti*: Biology and Ecology; Nelson MJ, editor. Washington, DC: Pan American Health Organization.

3. Diaz-Nieto LM, Macia A, Perotti MA, Beron CM (2013) Geographical limits of the Southeastern distribution of *Aedes aegypti* (Diptera, Culicidae) in Argentina. PLoS Negl Trop Dis 7: e1963.

4. Troyes L, Villegas Z, Troyes M (2006) Expansión del *Aedes aegypti* a localidades rurales de Cajamarca Rev Perú Med Exp Salud Pública 23: 163-167.

5. Halstead SB (2008) Dengue virus-mosquito interactions. Annu Rev Entomol 53: 273-291.

6. Ponnusamy L, Xu N, Stav G, Wesson DM, Schal C, et al. (2008) Diversity of bacterial communities in container habitats of mosquitoes. Microb Ecol 56: 593-603.

7. Kaufman MG, Walker ED, Smith TW, Merritt RW, Klug MJ (1999) Effects of larval mosquitoes (Aedes triseriatus) and stemflow on microbial community dynamics in container habitats. Appl Environ Microbiol 65: 2661-2673.

8. Ponnusamy L, Xu N, Nojima S, Wesson DM, Schal C, et al. (2008) Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by Aedes aegypti. Proc Natl Acad Sci U S A 105: 9262-9267.

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

10. Sota T, Mogi M (1992) Interspecific variation in desiccation survival time of *Aedes* (Stegomyia) mosquito eggs is correlated with habitat and egg size. Oecologia 90: 353-358.

11. Juliano SA, O'Meara GF, Morrill JR, Cutwa MM (2002) Desiccation and thermal tolerance of eggs and the coexistence of competing mosquitoes. Oecologia 130: 458-469.

12. Soper FL (1967) Dynamics of *Aedes aegypti* distribution and density. Seasonal fluctuations in the Americas. Bull World Health Organ 36: 536-538.

13. Guagliardo SA, Morrison AC, Barboza JL, Requena E, Astete H, et al. (2015) River boats contribute to the regional spread of the dengue vector Aedes aegypti in the Peruvian Amazon. PLoS Negl Trop Dis (in press).

14. Morrison AC, Sihuincha M, Stancil JD, Zamora E, Astete H, et al. (2006) Aedes aegypti (Diptera: Culicidae) production from non-residential sites in the Amazonian city of Iquitos, Peru. Ann Trop Med Parasitol 100 Suppl 1: S73-S86.

15. Guagliardo SA, Barboza JL, Morrison AC, Astete H, Vazquez-Prokopec G, et al. (2014) Patterns of geographic expansion of Aedes aegypti in the Peruvian Amazon. PLoS Negl Trop Dis 8: e3033.

16. Team RDC (2008) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Chapter 5: River Boats Drive *Aedes aegypti* Gene Flow in the Peruvian Amazon

5.1 Introduction

Anthropogenic activities such as trade and transportation contribute to the unintentional spread of invasive organisms across the globe [1-5], resulting in the potential for serious consequences for public health, the economy, and native ecosystems [6-12]. Pathogens and insect vectors of human diseases are important examples of invasive organisms that directly impact human health.

The invasive mosquito, *Aedes aegypti*, is the primary vector of dengue and urban yellow fever viruses, and has been shown to be a significant vector of Chikungunya virus [13,14], and a competent or suspected vector of Mayaro virus [15] and Venezuelan Equine Encephalitis [16]. Thought to be African in origin, *Ae. aegypti* most likely was transported to the Americas via ships used for the transport of slaves and goods in the 15th-19th centuries [17-19]. *Ae. aegypti* was first reported in Peru in 1852, and was declared eradicated in 1958 following the success of a large-scale Pan American Health Organization yellow fever control program [20]. The Amazonian city of Iquitos was the first site of *Ae. aegypti* (in 1984) and dengue (in 1990) reestablishment in Peru [21,22]. In recent years throughout Latin America and Peru, *Ae. aegypti* mosquitoes have been expanding geographically from urban to peri-urban and rural areas [23-25].

Ae. aegypti dispersal may occur in one of two ways: 1) The slower, active dispersal of adult female mosquitoes in search of a bloodmeal or oviposition sites, or 2) The faster, passive human-mediated dispersal, by which humans unintentionally transport mosquitoes via vehicle traffic (boats, cars, etc). The latter mechanism may involve the transport of eggs, larvae, pupae, or adult mosquitoes, all of which have been documented in vehicles, boats in particular [17,26-31]. Our previous research in the Peruvian Amazon demonstrated *Ae. aegypti* infestation on different vehicles commonly used for trade and transportation, including large barges, medium-sized barges, and buses [32] (**Chapter 3**). Understanding the relative role and importance of both mechanisms is paramount for better understanding of vector population structure and the dynamics of pathogen transmission.

Previous studies have characterized *Ae. aegypti* population structure at global scales [33-35], regions within countries [36,37], and within cities [38-40]. At various spatial scales, *Ae. aegypti* genetic differentiation is due to the vector's limited flight range (thought to not exceed 100m under natural conditions [41-44]), the application of insecticides, varying degrees of human population densities, and water storage habits (which may also contribute to *Ae. aegypti*'s capacity to transmit dengue and other viruses) [38,45,46]. At coarser scales, human transportation networks have indirectly been implicated as a driver of *Ae. aegypti* gene flow: relatedness between populations has been shown to loosely correlate with major highways and water ways [33]. Yet, to our knowledge, no studies to date have explicitly measured network connectivity, or linked observed patterns to possible mechanisms of long distance dispersal. In the present study, we use a panel of 10 microsatellites to characterize *Ae. aegypti* population structure in seven towns surrounding the Amazonian city of Iquitos, Peru. In contrast with previous work, we propose a novel method of measuring network distance through the Propagule Pressure Index, which combines frequency of travel and infestation probability of different vehicle types.

5.2 Methods

Study Area

Accessible only by plane and boat, Iquitos is surrounded by a number of other, smaller settlements that are primarily connected to one another via river networks. The only significant source of terrestrial transit is the 95km Iquitos-Nauta Highway, connecting Iquitos (pop: 406,340) to the smaller city of Nauta (pop: 13,983). Once the epicenter of the rubber industry in the early 1900s, Iquitos now relies predominantly on oil and timber exportations. This setting is ideal for studying the invasion dynamics of *Ae. aegypti*, as the region's inhabitants are dependent on both fluvial and terrestrial routes for trade and transportation.

Mosquito Sample Collections

Mosquito samples were collected from the city of Iquitos, and the towns of Nauta, Indiana, Mazan, Barrio Florida, Tamshiaco, and Aucayo. (**Table 5.1** shows characteristics associated with each town.) Adult and immature mosquitoes were collected either through aspiration of adults or through larval surveys. More than 20 individuals were collected for each sampling location, although mosquitoes collected from the towns of Indiana (N=18) and Mazan (N=14) were pooled into one population to ensure adequate sample sizes. (Our preliminary results indicated that these mosquito populations were genetically indistinguishable.)

Multiple samples were collected in the city of Iquitos in order to determine whether port mosquitoes in Iquitos were more closely related to mosquitoes in the surrounding towns (**Figure 5.1**). Iquitos A serves as a major hub of fluvial transit, harboring predominantly large barges (which usually consist of three floors and carry cargo and passengers throughout the Peruvian Amazon distances up to ~500km). Iquitos B is a secondary fluvial port, primarily harboring medium-sized barges that also carry cargo and passengers, but have only two floors and travel more locally to distances up to ~250km. We also sampled mosquitoes from the interior of Iquitos (Iquitos C) and from a recently urbanized neighborhood toward the Iquitos-Nauta highway (Iquitos D).

DNA Extraction and Amplification

The methods we used for genotyping mosquitoes with microsatellite markers are described in Wong et al. 2012 [47]. Genomic DNA from the mosquito body was purified by potassium acetate/ethanol precipitation [48]. In a multiplex PCR, we amplified 10 previously described microsatellite markers [49,50]). PCR products were diluted 1:60 and 1:40 in ddH_2O, respectively, and submitted to the UCD College of Agriculture and Environmental Sciences Genomics Facility (http://cgf.ucdavis.edu/home/) for fragment analysis on an ABI 3730 XL capillary electrophoresis sequencer (Life Technology Corp.). ABI Peak

Scanner™ software (Applera Corp., Norwalk, CT) was used to visualize resulting chromatograms. GS600 LIZ size standard (Life Technology Corp.) was included with each sample to determine the size of individual peaks. After identifying fragments, alleles were assigned using the MsatAllele package of R [51,52].

Microsatellite Data Analysis

Data analysis was carried out in Arlequin version 3.11 and Structure 2.3.4. Samples were tested for Hardy-Weinberg equilibrium and F_{ST} was calculated. Mantel tests were used to test genetic isolation by three different distance models. We used Euclidean distance (the shortest straight-line distance between two locations), fluvial path distance (the river route between towns), and shortest path distance (the shortest accessible fluvial or terrestrial route between two locations). For isolation by distance plots, we combined Iquitos populations into a single population and F_{ST} values were recalculated.

We also developed a "Propagule Pressure Index", combining the probability of *Ae. aegypti* infestation in different vehicle types with the frequency of travel between Iquitos and surrounding towns. *Ae. aegypti* infestation probabilities were calculated from our previous research (**Chapter 3**) documenting *Ae. aegypti* infestation across different vehicle types and specific in the city of Iquitos. In 2013, we conducted thorough entomological surveys on six different vehicle types commonly found in Iquitos: large barges, medium-sized barges, water taxis, speed boats, buses, and taxis. Our results showed that some vehicle types were consistently infested with *Ae. aegypti* across multiple months (71% of large barges, 35% of medium-sized barges, and 12.5% of buses). Simultaneous with entomological surveys, we interviewed vehicle drivers to determine the frequency of travel between Iquitos and surrounding towns for each vehicle type (and thus we were able to estimate the number of vehicles traveling to each town). Thus, the Propagule Pressure Index β is calculated as follows:

$$\beta = \Sigma (\gamma_i \theta_i)$$

Where:

γ_i = probability of infestation

θ_i = number of trips from Iquitos to surrounding towns

i represents different vehicle types (large barges, medium barges, and buses)

Values of β were plotted against F_{ST}, although no statistical tests were conducted due to low sample sizes. (Pairwise calculations of β were only available between Iquitos and other towns, leaving a total of five observations.)

Lastly, the program Structure was used to estimate the probability of each individual belonging to a haplotype group. We modeled K=1 haplotype groups up to K=15 haplotype groups, using 10,000 Markov Chain Monte Carlo runs, with a burn-in length of 10,000 additional runs. Credible sets were calculated and plotted for each value of K. The best value for K was determined by the narrowest credible set. Wilcoxon tests were performed to determine whether K groups significantly differed from one another.

5.3 Results

Of 90 tests, 62 (~70%) of loci from each population were found to be in Hardy-Weinberg equilibrium (**Table 5.2**). After applying a Sidak correction for multiple comparisons, significant heterozygous deficits were detected in all loci in Tamshiaco samples, Nauta (A10, AC5, AG2, AG3, AT1, B07), Aucayo (AG1), Barrio Florida (AG1, B07), Indiana/ Mazan (AG3), Iquitos B (AG1, AG3), Iquitos C (AC1, AC5, AG1, AG3), and Iquitos D (AG1, AG3, B07).

Pairwise F_{ST} values demonstrated significant but low to moderate differentiation for the majority of site pairs, after adjustment for multiple comparisons (**Table 5.3**). Comparisons involving samples from Barrio Florida showed significant differentiation for all site pairs, with F_{ST} values ranging from 0.077 to 0.15. Comparisons involving samples from Iquitos were significant in 13 of 25 comparisons. With the exception of comparisons made between Barrio Florida and Iquitos, Iquitos samples showed the least amount of genetic isolation for all site pairs with F_{ST} values ranging from 0.028 to 0.072. Notably, on average, mosquitoes collected from Iquitos B (the center for regional fluvial transit) had lower F_{ST} values than mosquitoes collected from the interior of Iquitos (Iquitos C and D), indicating more gene flow between mosquitoes from surrounding towns and those from Iquitos B. Within Iquitos, the comparison between Iquitos C (interior) and Iquitos D (recently urbanized) revealed a low but significant F_{ST} of 0.028.

There was no clear relationship between genetic distance (F_{ST}) and geographic distance for any of the three models (**Figure 5.3**, Euclidean model: Mantel p-value=0.575; Fluvial path model p=0.511; Shortest path model; p=0.501). A negative correlation between genetic distance and network distance, as measured by the Propagule Pressure Index, was observed, although it was not possible to test this statistically due to low sample sizes.

Results from the Bayesian cluster analysis showed a clear pattern of genetic admixture from the sample populations. The most likely number of population groups was K=5 or K=6 (**Figure 5.4**). Barrio Florida mosquitoes showed dominance by a particular group, whereas Iquitos populations showed similar composition of population group as that of surrounding towns. Within Iquitos, our Structure results also showed a similar composition of population group membership for the two port populations (IQT A and B) as the recently urbanized site (IQT D). Interestingly, mosquito collections from the interior of Iquitos, IQT C, showed a different pattern of percent population membership.

5.4 Discussion

Both ecological [23-25] and population genetics studies [33,53,54] have pointed to human transportation networks as a major driver of *Ae. aegypti* spread, yet, to our knowledge, none of these studies have linked ecological evidence with transportation data and population genetics data. In this study we have proposed a new method integrating this data. This is particularly relevant in evaluating isolation by distance in scenarios where geographic distance is not an adequate predictor of genetic distance. The Propagule Pressure Index takes into account both vehicle infestation rates and the frequency of vehicle traffic between two locations. Infestation rates across vehicle types are heterogeneous (**Chapter 3**), and therefore the type of vehicles traveling in a region should be taken into account.

Ae. aegypti regional gene flow

Gene flow between Iquitos and surrounding towns was greatest relative to all other comparisons, as demonstrated by low pairwise F_{ST} values. This is logical since Iquitos serves the major transportation hub within the Peruvian Amazon, with frequent visits of barges and other types of boats from surrounding areas. Our results suggest a regional metapopulation structure with source-sink dynamics, signifying that introductions from Iquitos to surrounding towns occur regularly. Notably, F_{ST} values for a regional port (Iquitos B), were overall lower than pairwise F_{ST} values collected from the interior of Iquitos (Iquitos C and D), indicating that more population mixing occurs between port mosquito populations and surrounding towns than between interior populations and surrounding towns. In contrast, Barrio Florida mosquito populations were genetically isolated in comparison with other towns. Barrio Florida is a small town (human population ~800), and our interview data revealed that this town receives almost no traffic from vehicles likely to be infested with *Ae. aegypti* such as large and medium-sized barges.

Consistent with findings from other studies, our comparisons of genetic distance and geographic distance did not reveal a pattern of isolation by distance. Other research from Peru (using mitochondrial DNA markers) showed that nucleotidic diversity was not related to geographic distance when comparing mosquito populations from Iquitos, Lima, and from the coastal city of Piura [55]. Elsewhere in Latin America (in Mexico and Brazil using mtDNA markers) similar discordance between genetic and geographic distance has been observed [37,53]. Although isolation by distance was shown at a much coarser scale in the Brazilian Amazon, [54], when the authors excluded the most extreme observations (the most geographically distant populations) from the analysis, isolation by distance was not observed. Taken together, results from these studies suggest that A) *Ae. aegypti* long distance dispersal is most certainly due to human transportation networks, B) Isolation by distance for *Ae. aegypti* populations is observed at coarser scales, and C) The most relevant types of human transit (riverine, terrestrial, aerial) for *Ae. aegypti* long distance dispersal depend on the spatial and temporal scales at which these questions are examined.

Ae. aegypti *gene flow within cities*

Previous research has explored the relationship between urban landscape structures and *Ae. aegypti* dispersal within cities. Hemme et al 2010 [56] showed that highways could act as barriers to dispersal, while Reiter [57] suggested that buildings do not impede dispersal. In our study, within Iquitos we observed that *Ae. aegypti* population group membership of port mosquitoes and recently urbanized mosquitoes were similar, while interior mosquitoes had a different pattern of population membership. This pattern could be explained by the movement of trucks along major roadways within Iquitos. That is, goods and materials for construction arrive to Iquitos ports from elsewhere in the Peruvian Amazon, and large trucks carry these goods from ports to areas within the city experiencing rapid development. Further study is required to A) determine whether trucks are frequently infested with *Ae. aegypti*, B) characterize truck movement patterns within Iquitos, and C) determine which *Ae. aegypti* life stage is most important in its dispersal.

As urbanization and trade continues to increase across South America, *Ae. aegypti* mosquitoes are likely to continue to spread to new locations, thus facilitating new dengue outbreaks. Knowledge about the continued invasion of *Ae. aegypti* mosquitoes will aid in the prevention of its spread and resulting disease.

5.5 Tables

Table 5.1. Characteristics of Study Sites. We collected >20 individuals per sampling location, with multiple sampling sites within Iquitos. Human population data was derived from the Peruvian National Census in 2007.

City	Sub-Sample	N	Collection Date	Human population
Aucayo	-	20	Sept 2008	806
Barrio Florida	-	31	April 2008	728
Indiana-Mazan	-	32	Mar 2008	6,594
Nauta		55	May 2007, Mar 2008	13,983
Tamishaco	-	76	Mar 2008	4,583
Iquitos	-	-	-	406,340
	Iquitos A - Port	21	May 2007, Mar 2008	-
	Iquitos B- Port	40	Mar 2008	-
	Iquitos C – Interior	29	May 2007; Feb, Mar 2008	-
	Iquitos D - Recently urbanized	35	Feb 2008	-

Table 5.2. Summary of variation at 10 microsatellite loci by sampling location. N, number of individuals; FIS, inbreeding coefficient. Bold denotes significant departure from HW after Sidak correction.

Locus		**Aucayo**	**Barrio Florida**	**Indiana/ Mazan**	**Nauta**
A10	FIS	0.231	0.1327	0.061	**0.327**
	N	20	26	32	55
AC1	FIS	0.209	0.200	0.131	0.423
	N	17	25	30	32
AC5	FIS	-0.041	0.294	0.100	**0.111**
	N	17	25	30	55
AG1	FIS	**0.145**	**0.383**	0.231	0.193
	N	20	28	32	55
AG2	FIS	0.6	0.308	-0.194	**0.154**
	N	17	26	30	55
AG3	FIS	0.396	0.143	**0.936**	**0.731**
	N	17	25	30	32
AG5	FIS	0.058	0.143	0.029	0.085
	N	20	29	32	55
AT1	FIS	0.242	0	0.296	**0.292**
	N	20	27	32	55
B07	FIS	0.325	**0.774**	0.308	**0.478**
	N	15	26	30	51
H08	FIS	0.302	0	0.077	0.029
	N	20	26	31	55

Table 5.2 Continued

Locus		**Tamishiaco**	**Iquitos A**	**Iquitos B**	**Iquitos C**	**Iquitos D**
A10	FIS	**0.4**	-0.021	-0.107	-0.077	0.238
	N	73	21	37	32	28
AC1	FIS	**0.312**	0.459	0.072	**0.567**	0.283
	N	59	12	39	35	23
AC5	FIS	**0.472**	0.294	0.319	**0.561**	0.205
	N	58	15	39	34	26
AG1	FIS	**0.481**	0.348	**0.411**	**0.556**	**0.270**
	N	71	21	37	31	28
AG2	FIS	0.222	0.217	-0.109	0.211	0.186
	N	61	15	39	34	26
AG3	FIS	**0.493**	1	**0.814**	**0.846**	**0.875**
	N	62	12	39	34	23
AG5	FIS	**0.321**	0.024	0.117	0.141	0.117
	N	73	21	37	33	28
AT1	FIS	**0.321**	0.230	-0.013	0.027	0.260
	N	75	21	37	32	28
B07	FIS	**0.887**	0.135	0.478	0.471	**0.527**
	N	57	19	37	27	27
H08	FIS	**0.431**	-0.170	0.400	0.370	0.152
	N	76	21	37	32	28

Table 5.3. Pairwise FST Table.

	Aucayo	Barrio Florido	Indiana/ Mazan	Nauta	Tamshiaco
Aucayo	0				
Barrio Florida	**0.12467**	0			
Indiana/Mazan	**0.04544**	**0.12859**	0		
Nauta	**0.07151**	**0.13629**	**0.05976**	0	
Tamshiaco	**0.03449**	**0.07745**	**0.05357**	**0.04845**	0
Iquitos A	0.03864	**0.15392**	0.02052	**0.07231**	**0.04147**
Iquitos B	0.03666	**0.09661**	0.01912	**0.04217**	0.01124
Iquitos C	**0.04173**	**0.14367**	**0.04644**	**0.06495**	**0.02812**
Iquitos D	**0.04403**	**0.10716**	0.00076	0.02565	0.02812

Table 5.3 Continued

	Iquitos A	**Iquitos B**	**Iquitos C**	**Iquitos D**
Aucayo				
Barrio Florida				
Indiana/Mazan				
Nauta				
Tamshiaco				
Iquitos A	0			
Iquitos B	0.00053	0		
Iquitos C	0.00789	0.00711	0	
Iquitos D	0.01223	0.00594	**0.02862**	0

5.6. Figures

Figure 5.1. Map of study collection sites. We collected mosquitoes from seven population centers in the Peruvian Amazon: Iquitos, Nauta, Tamshiaco, Barrio Florida, Indiana, and Mazan. Collections from Indiana and Mazan were pooled into a single group to ensure >20 individuals per site. In addition, we collected mosquitoes from four locations within Iquitos, two port sites and two interior sites.

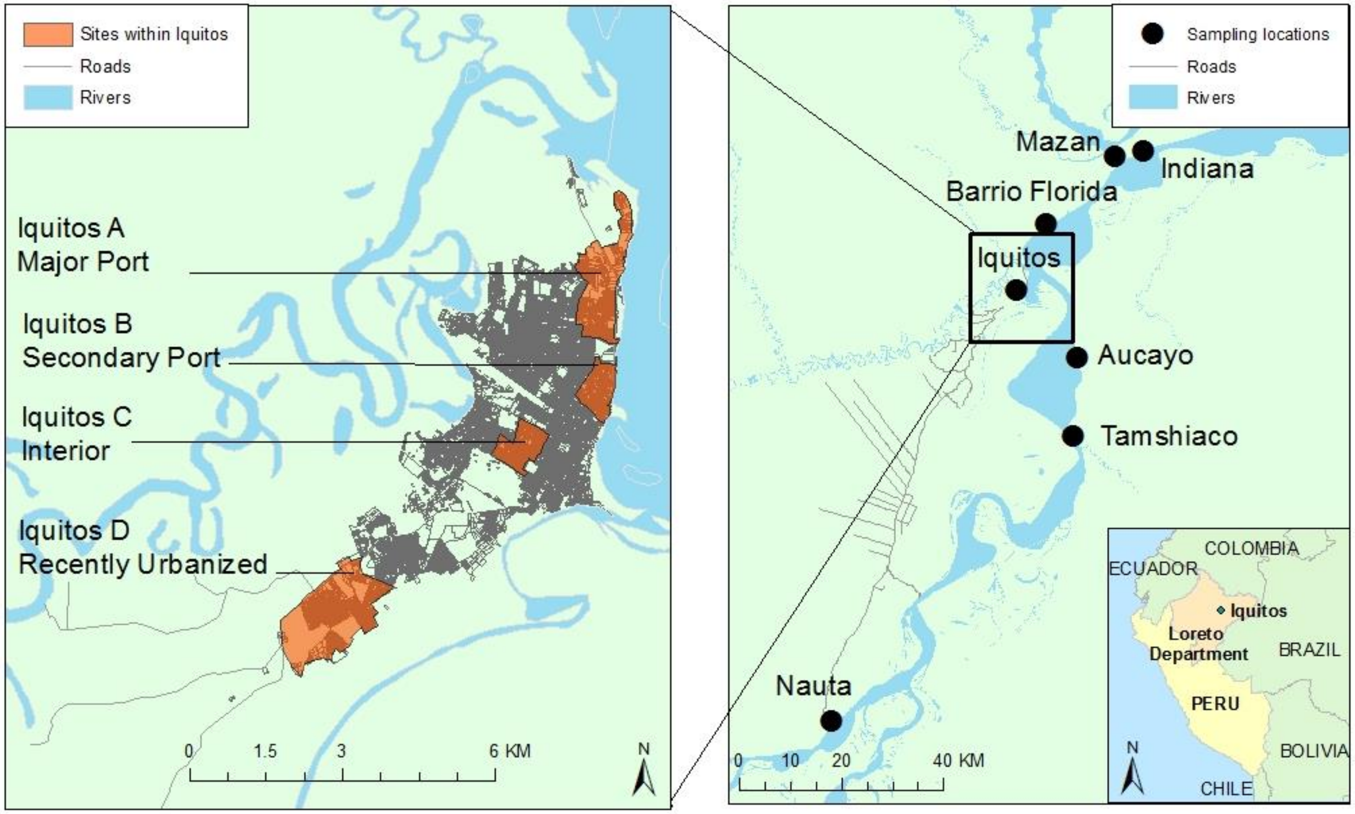


Figure 5.2. Geographic distance models. We used three different models of geographic distance to evaluate isolation by distance including A) Euclidean distance – the most direct straight-line distance between locations, B) Fluvial path distance – the river path between locations, and C) Shortest path distance – the shortest combination of terrestrial and fluvial routes that connect locations.

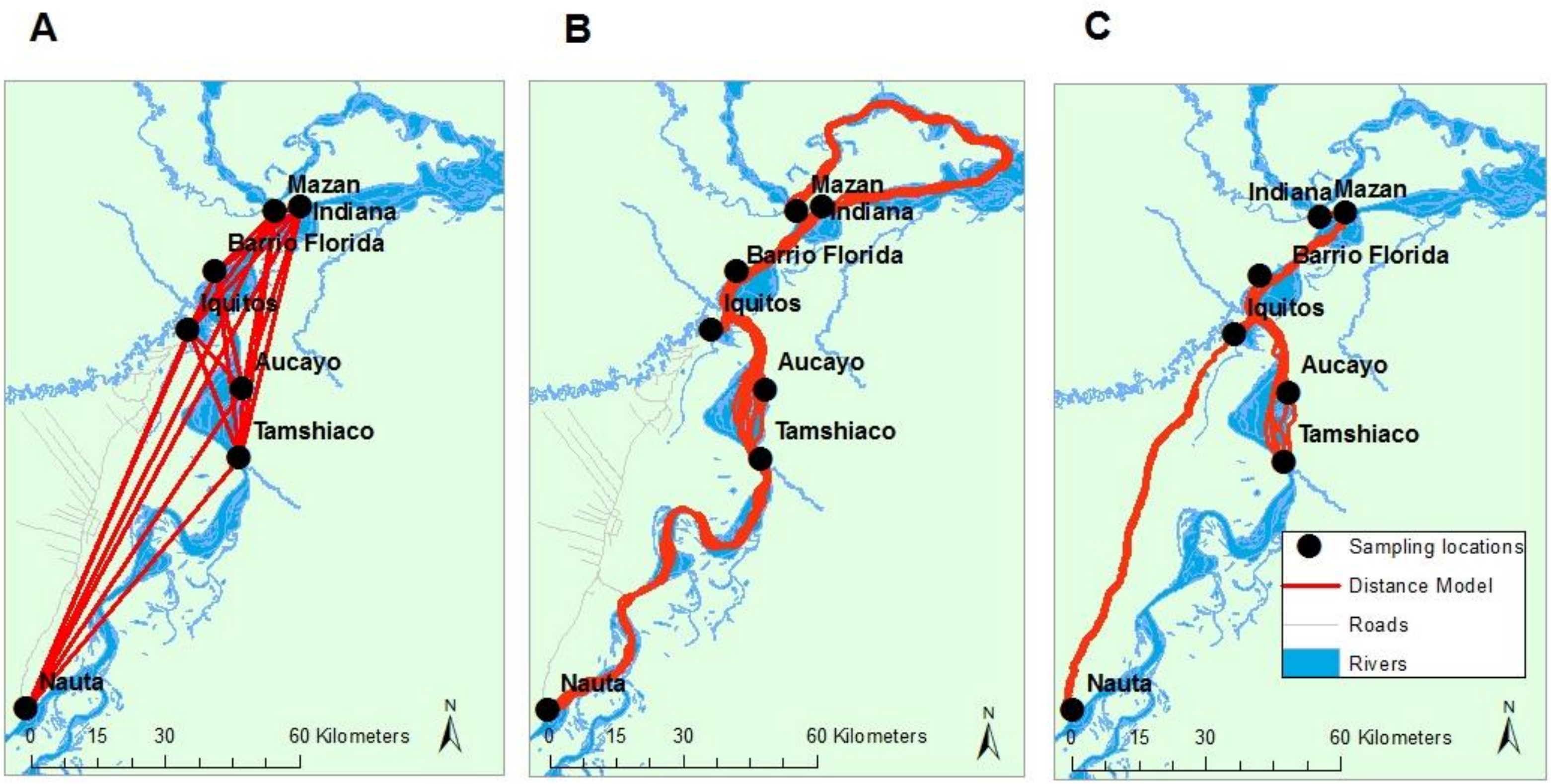


Figure 5.3. Isolation by distance models for three measures of geographic distance and for one measure of network distance. (P-values shown are Mantel probabilities.)

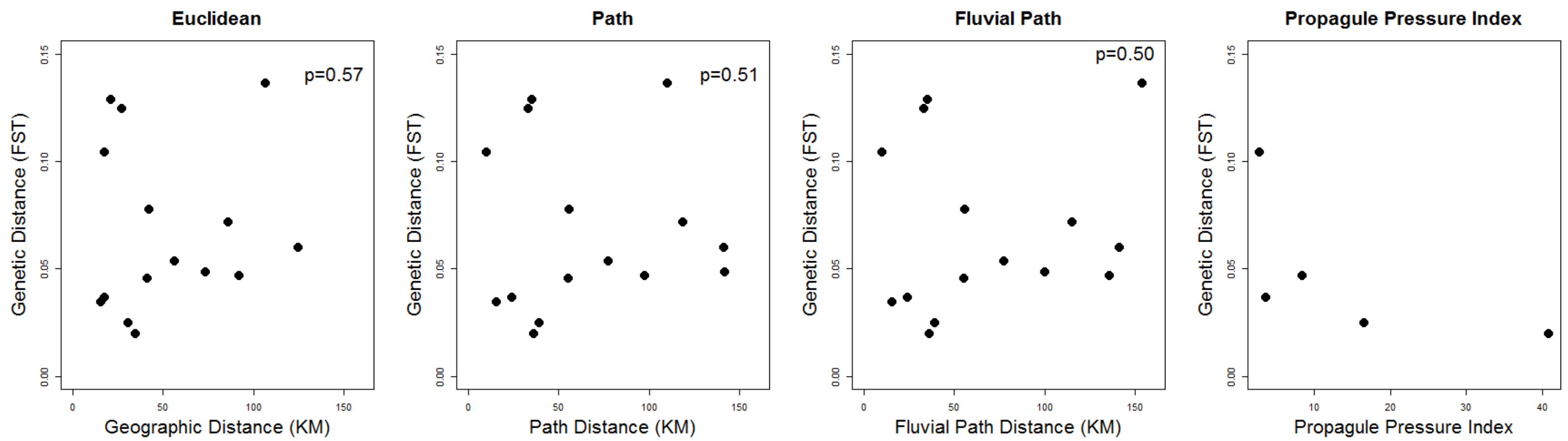


Figure 5.4. Structure Diagrams. The most likely number of populations were identified as being K=5 or K=6 different groups.

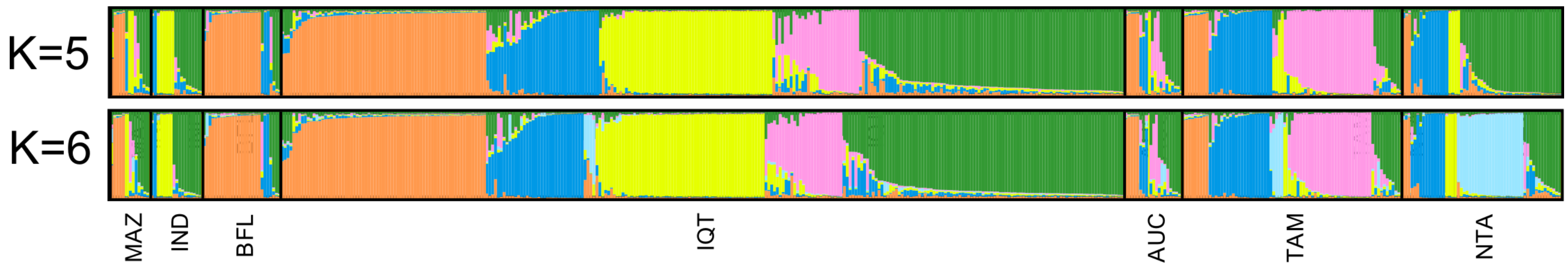
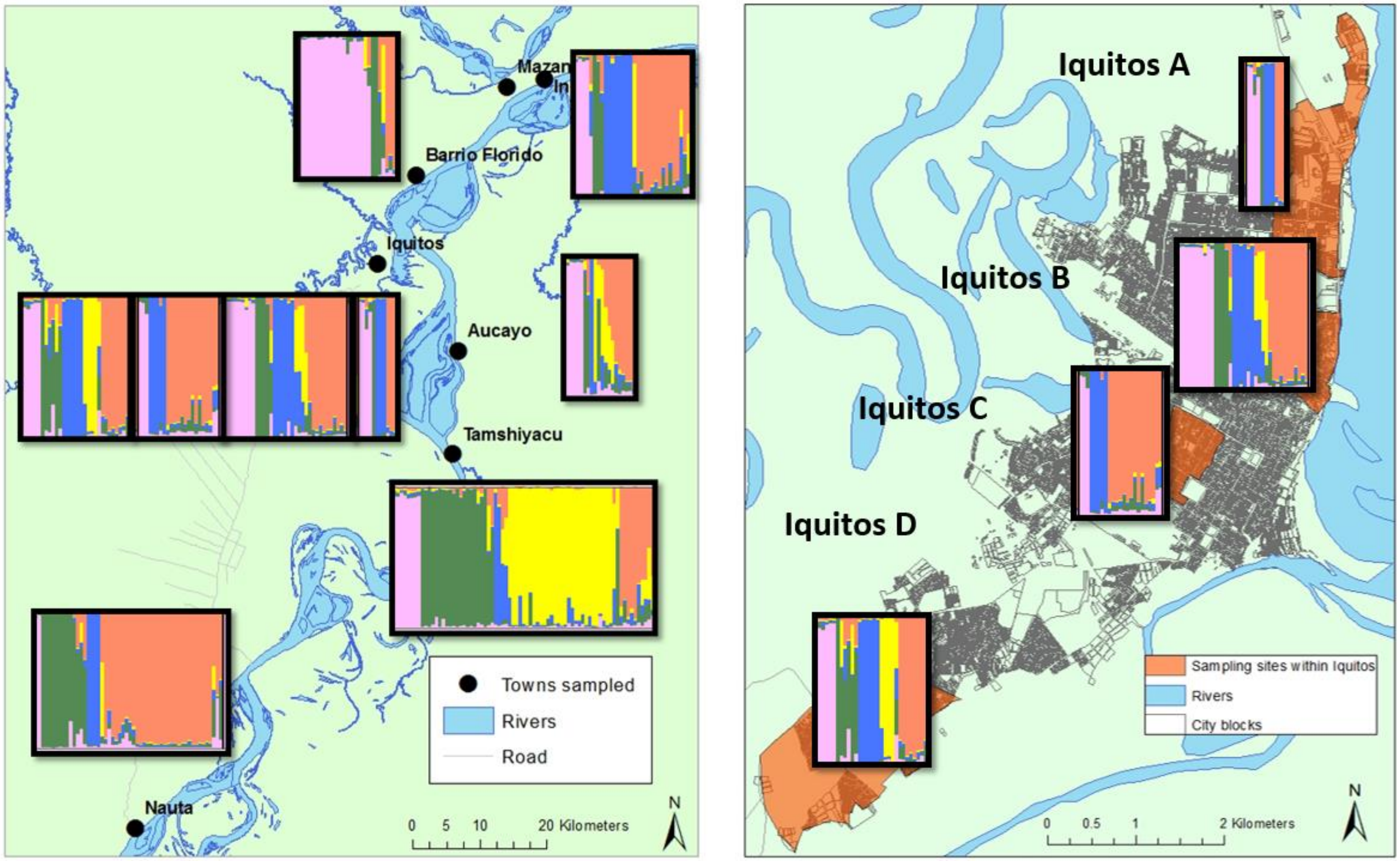


Figure 5.5. Structure results superimposed on maps.



5.7 References Cited

1. Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. J Appl Ecol 46: 10–18.

2. Lockwood JL, Cassey P, Blackburn T (2005) The role of propagule pressure in explaining species invasions. Trends Ecol Evol 20: 223–228.

3. Lodge DM, Williams S, MacIsaac HJ, Hayes KR, Leung B, et al. (2006) Biological invasions: recommendations for U.S. policy and management. Ecol Appl 16: 2035-2054.

4. McNeely J (2006) As the world gets smaller, the chances of invasion grow. Euphytica 148: 5-15.

5. Meyerson LA, Moooney HA (2007) Invasive alien species in an era of globalization. Front Ecol Environ 5: 199-208.

6. Vitousek P, DAntonio C, Loope L, Westbrooks R (1996) Biological invasions as global environmental change. Am Sci 84: 468–478.

7. Wilcove D, Rothstein D, Dubow J, Phillips A (1998) Quantifying threats to imperiled species in the United States. Biosci Rep 48: 607–615.

8. Sala OE, Chapin FS, Armesto JJ, al e (2000) Biodiversity - Global biodiversity scenarios for the year 2100. Science 287.

9. Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ 52: 273-288.

10. Sax DF, Stachowicz JJ, Gaines SD (2005). Sunderland, MA: Sinauer Associates Inc.

11. Elton CS (1958) The ecology of invasions by animals and plants London: Methuen.

12. Sakai AK, Allendorf FW, Holt JS, Lodge DM, Molofsky J, et al. (2001) The population biology of invasive species. Annu Rev Ecol Syst 32 305–332.

13. Dubrulle M, Mousson L, Moutailler S, Vazeille M, Failloux AB (2009) Chikungunya virus and Aedes mosquitoes: saliva is infectious as soon as two days after oral infection. PLoS One 4: e5895.

14. Mourya DT, Thakare JR, Gokhale MD, Powers AM, Hundekar SL, et al. (2001) Isolation of chikungunya virus from Aedes aegypti mosquitoes collected in the town of Yawat, Pune District, Maharashtra State, India. Acta Virol 45: 305-309.

15. Long KC, Ziegler SA, Thangamani S, Hausser NL, Kochel TJ, et al. (2011) Experimental transmission of Mayaro virus by Aedes aegypti. Am J Trop Med Hyg 85: 750-757.

16. Larsen JR, Ashley RF (1971) Demonstration of Venezuelan equine encephalomyelitis virus in tissues of Aedes Aegypti. Am J Trop Med Hyg 20: 754-760.

17. Soper FL (1967) Dynamics of *Aedes aegypti* distribution and density. Seasonal fluctuations in the Americas. Bull World Health Organ 36: 536-538.

18. Gubler DJ (1997) Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. Dengue and dengue hemorrhagic fever. Cambridge, UK CAB International pp. 1-22.

19. Soper FL, Wilson DB, Lima S, Antunes W (1943) The organization of permanent nationwide anti-*Aedes aegypti* measures in Brazil. New York: The Rockefeller Foundation.

20. Severo OP (1958) Evolución de la campaña anti-Aegyti en los últimos diez años. Bol Of Sanit Panam 45: 375-386.

21. Phillips I, Need J, Escamilla J, Colan E, Sanchez S, et al. (1992) First documented outbreak of dengue in the Peruvian Amazon region. Bull Pan Am Health Organ 26: 201-207.

22. Andrade CS, Caceres AG, Vaquerizo A, Ibanez-Bernal S, Cachay LS (2001) Reappearance of Aedes aegypti (Diptera: Culicidae) in Lima, Peru. Mem Inst Oswaldo Cruz 96: 657-658.

23. Diaz-Nieto LM, Macia A, Perotti MA, Beron CM (2013) Geographical limits of the Southeastern distribution of *Aedes aegypti* (Diptera, Culicidae) in Argentina. PLoS Negl Trop Dis 7: e1963.

24. Troyes L, Villegas Z, Troyes M (2006) Expansión del *Aedes aegypti* a localidades rurales de Cajamarca Rev Perú Med Exp Salud Pública 23: 163-167.

25. Guagliardo SA, Barboza JL, Morrison AC, Astete H, Vazquez-Prokopec G, et al. (2014) Patterns of geographic expansion of Aedes aegypti in the Peruvian Amazon. PLoS Negl Trop Dis 8: e3033.

26. Souchan E (1902) On the transport of mosquitoes by vessels. Med Rec 62: 8-9.

27. Christopher SR (1960) *Aedes aegypti* (L.), the yellow fever mosquito. Its life history, bionomics and structure. . Cambridge, UK: Cambridge University Press.

28. Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-496.

29. Chadee DD (1984) *Aedes aegypti* aboard boats at Port of Spain, Trinidad, W.I. (1972–1982). . Mosquito News 44: 1–3.

30. Hughes JH, Porter JE (1956) Dispersal of mosquitoes through transportation, with particular reference to immature stages. . Mosq News 16: 106-111.

31. Sukehiro N, Kida N, Umezawa M, Murakami T, Arai N, et al. (2013) First report on invasion of yellow fever mosquito, Aedes aegypti, at Narita International Airport, Japan in August 2012. Jpn J Infect Dis 66: 189-194.

32. Morrison AC, Sihuincha M, Stancil JD, Zamora E, Astete H, et al. (2006) Aedes aegypti (Diptera: Culicidae) production from non-residential sites in the Amazonian city of Iquitos, Peru. Ann Trop Med Parasitol 100 Suppl 1: S73-S86.

33. Huber K, Loan LL, Chantha N, Failloux AB (2004) Human transportation influences *Aedes aegypti* gene flow in Southeast Asia. Acta Trop 90: 23-29.

34. Tabachnick WJ, Powell JR (1979) A world-wide survey of genetic variation in the yellow fever mosquito, Aedes aegypti. Genet Res 34: 215-229.

35. Powell JR, Tabachnick WJ, Arnold J (1980) Genetics and the origin of a vector population: Aedes aegypti, a case study. Science 208: 1385-1387.

36. Apostol BL, Black WCt, Reiter P, Miller BR (1994) Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of Aedes aegypti families at oviposition sites in San Juan, Puerto Rico. Am J Trop Med Hyg 51: 89-97.

37. Gorrochotegui-Escalante N, Munoz ML, Fernandez-Salas I, Beaty BJ, Black WCt (2000) Genetic isolation by distance among Aedes aegypti populations along the northeastern coast of Mexico. Am J Trop Med Hyg 62: 200-209.

38. Huber K, Le Loan L, Hoang TH, Ravel S, Rodhain F, et al. (2002) Genetic differentiation of the dengue vector, Aedes aegypti (Ho Chi Minh City, Vietnam) using microsatellite markers. Mol Ecol 11: 1629-1635.

39. Paupy C, Chantha N, Vazeille M, Reynes JM, Rodhain F, et al. (2003) Variation over space and time of Aedes aegypti in Phnom Penh (Cambodia): genetic structure and oral susceptibility to a dengue virus. Genet Res 82: 171-182.

40. Paupy C, Chantha N, Huber K, Lecoz N, Reynes JM, et al. (2004) Influence of breeding sites features on genetic differentiation of Aedes aegypti populations analyzed on a local scale in Phnom Penh Municipality of Cambodia. Am J Trop Med Hyg 71: 73-81.

41. Morlan HB, Hayes RO (1958) Urban dispersal and activity of *Aedes aegypti*. Mosq News: 137-144.

42. Muir LE, Kay BH (1998) *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. Am J Trop Med Hyg 58: 277-282.

43. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg 72: 209-220.

44. Edman JD, Scott TW, Costero A, Morrison AC, Harrington LC, et al. (1998) Aedes aegypti (Diptera: Culicidae) movement influenced by availability of oviposition sites. J Med Entomol 35: 578-583.

45. Tran KT, Vazeille-Falcoz M, Mousson L, Tran HH, Rodhain F, et al. (1999) Aedes aegypti in Ho Chi Minh City (Viet Nam): susceptibility to dengue 2 virus and genetic differentiation. Trans R Soc Trop Med Hyg 93: 581-586.

46. Huber K, Loan LL, Hoang TH, Tien TK, Rodhain F, et al. (2002) Temporal genetic variation in Aedes aegypti populations in Ho Chi Minh City (Vietnam). Heredity (Edinb) 89: 7-14.

47. Wong J, Morrison AC, Stoddard ST, Astete H, Chu YY, et al. (2012) Linking oviposition site choice to offspring fitness in *Aedes aegypti*: consequences for targeted larval control of dengue vectors. PLoS Negl Trop Dis 6: e1632.

48. Black WC, DuTeau NM (1997) RAPD-PCR and SSCP analysis for insect population genetic studies. In: Crampton J, Beard CB, Louis C, editors. The molecular biology of disease vectors: a methods manual. New York: Chapman & Hall. pp. 361-371.

49. Chambers EW, Meece JK, McGowan JA, Lovin DD, Hemme RR, et al. (2007) Microsatellite isolation and linkage group identification in the yellow fever mosquito Aedes aegypti. J Hered 98: 202-210.

50. Slotman MA, Kelly NB, Harrington LC, Kitthawee S, Jones JW, et al. (2007) Polymorphic microsatellite markers for studies of Aedes aegypti (Diptera: Culicidae), the vector of dengue and yellow fever. Molecular Ecology Notes 7: 168-171.

51. Alberto F (2013) MsatAllele: Visualizes the scoring and binning of microsatellite fragment sizes. R package version 1.05 ed.

52. R Development Core Team (2008) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

53. da Silva AG CI, Santos WS, Luz LB, Ribolla PEM, Abad-Franch F (2012) Gene flow networks among American Aedes aegypti populations. Evolutionary Applications 5: 664-676.

54. Lima RS, Jr., Scarpassa VM (2009) Evidence of two lineages of the dengue vector Aedes aegypti in the Brazilian Amazon, based on mitochondrial DNA ND4 gene sequences. Genet Mol Biol 32: 414-422.

55. da Costa-da-Silva AL, Capurro ML, Bracco JE (2005) Genetic lineages in the yellow fever mosquito Aedes (Stegomyia) aegypti (Diptera: Culicidae) from Peru. Mem Inst Oswaldo Cruz 100: 539-544.

56. Hemme RR, Thomas CL, Chadee DD, Severson DW (2010) Influence of urban landscapes on population dynamics in a short-distance migrant

mosquito: evidence for the dengue vector *Aedes aegypti*. PLoS Negl Trop Dis 4: e634.

57. Reiter P, Amador MA, Anderson RA, Clark GG (1995) Short report: dispersal of Aedes aegypti in an urban area after blood feeding as demonstrated by rubidium-marked eggs. Am J Trop Med Hyg 52: 177-179.

Chapter 6: Conclusion

6.1 Summary

In **Chapter 2**, we showed that although riverine communities and road communities were equally likely to have *Ae. aegypti* mosquitoes, the spatial distribution of infestation along rivers was much further from Iquitos in comparison with its distribution along the Iquitos-Nauta highway. Probability of *Ae. aegypti* infestation was positively associated with community proximity to Iquitos and large human populations. Within infested communities, households more likely to contain *Ae. aegypti* had more passively-filled containers and co-occurrence of urban mosquitoes of other genera. At the container scale, large water tanks and drums with solar exposure were most likely to have *Ae. aegypti* mosquitoes. The chief novel finding of this study was at the community scale - larger communities that are proximal to cities are more likely to be infested with *Ae. aegypti*. Information about human population size and community location is usually readily available (and inexpensive to collect). Accordingly, resource-constrained local health departments can easily calculate risk based on these two risk factors. Further, this finding demonstrates that gravity models, which assume invasion risk to be a function of “attraction” and the inverse distance) are likely to be an effective way of modelling *Ae. aegypti* spread, potentially on a range of spatial scales from local to global.

Results from **Chapter 3** showed *Ae. aegypti* infestation on several vehicle types commonly found in the Iquitos region, including large barges, medium barges, and buses. No *Ae. aegypti* mosquitoes were found on speed boats, water taxis, or taxis. Large barges (71.9% infested) and medium barges (39.4% infested) accounted for most of the infestations. Buses had an overall infestation rate of 12.5%. On large barges, the greatest number of *Ae. aegypti* adults were found in October, whereas most immatures were found in February followed by October. The vast majority of larvae (85.9%) and pupae (76.7%) collected in large barges were produced in puddles formed in cargo holds. Larges barges provide suitable habitats for mosquitoes at all life stages – they contain dark, damp cargo storage spaces for adult resting sites, human hosts for blood meals, and ample oviposition sites. Although buses travel more frequently (up and down the Iquitos-Nauta highway), river boats carry more mosquitoes and travel further distances. From this, we conclude that river boats likely serve as the most important contributors to mosquitoes' propagule pressure over long distances throughout the Peruvian Amazon, where riparian corridors are the main route of transport.

Our semi-natural experiment with baited ovitraps (**Chapter 4**) showed that *Ae. aegypti* oviposition on boats occurs during travel, and that oviposition occurs consistently throughout the year. Further, not only do a small proportion of boats produce the majority of adult and immature *Ae. aegypti*, but a small proportion of traps were responsible for the majority of larval production.

Lastly, in **Chapter 5**, we used population genetics tools to demonstrate that gene flow among *Ae. aegypti* populations in the Peruvian Amazon is correlated with boat traffic. Our calculations of F_{ST} showed moderate genetic differentiation between many site pairs, with the town of Barrio Florida showing the greatest degree of differentiation. Populations from Iquitos were closely related to all other sites, which is logical since Iquitos serves as the major transportation hub in the Peruvian Amazon. Isolation by distance was evaluated using three measures of geographic distance (Euclidean, path, and fluvial path). No correlation was observed between genetic distance and geographic distance for any distance measure. A Propagule Pressure Index (combing the probability of vehicle infestation and frequency of travel as a measure of network distance) showed that *Ae. aegypti* gene flow among sub-populations is greatest between locations with heavy boat traffic such as Iquitos-Nauta (which also has heavy road traffic) and Iquitos-Indiana-Mazan, and lowest between locations with little or no boat (or road) traffic such as Barrio Florida-Iquitos. Bayesian clustering analysis showed definite admixture, with 5-6 genetic clusters. Our results strongly support the hypothesis that human transportation networks, especially via boats, are responsible for *Ae. aegypti* spread in the Peruvian Amazon.

Taken together, evidence presented from this dissertation highlights the importance of river boats as a major driver of *Ae. aegypti* regional expansion in the Peruvian Amazon. Although past research has pointed to large ocean barges that travel intercontinentally as a vehicle for the long-distance transport of mosquitoes (and invasive species in general), here we have added the following observations about *Ae. aegypti* invasion: 1) Community-level risk for invasion is a function of connectivity and proximity to major urban centers, 2) Other vehicle types are also responsible for transporting mosquitoes (such as buses and Amazonian barges, which are much smaller that ocean barges), 3) In addition to immature mosquitoes, human vehicles also transport adult mosquitoes, and 4) Few individual vehicles produce the majority of mosquitoes.

6.2 Further Research

The principal findings of this dissertation and remaining knowledge gaps are summarized in **Figure 6.1**.

In **Chapter 2**, we identified risk factors for *Ae. aegypti* infestation at the community, household, and container scales. In the community scale univariable logistic regression model, the use of river/ stream water reduced the odds of *Ae. aegypti* establishment. This finding, however, was not significant in the multivariable model, likely due to the small number of observations in our dataset and low statistical power. Still, the association between water use and *Ae. aegypti* establishment could be further explored through the testing of three main hypotheses: 1) Water type may be correlated with other factors important for *Ae. aegypti* establishment and spread. (For example, piped water systems are likely to be most abundant in larger settlements closer to Iquitos city.) 2) River/stream water may be less attractive to *Ae. aegypti* mosquitoes for oviposition due to the chemical and organic composition of the water. 3) Containers filled with river/stream water may be frequently emptied and re-filled, thus reducing the probability of the accumulation of organic material and therefore oviposition. For the first mechanism, more extensive datasets that include information on water use and other risk factors like proximity to urban centers could be used to repeat the analysis. (Notably, we were unable to identify a significant correlation between river/stream water usage and population size or distance to Iquitos, **Figure S2.1**.) The second mechanism could be evaluated through simple oviposition experiments to test the hypothesis that river water is less suitable for *Ae. aegypti* oviposition and development. Lastly, longitudinal studies could elucidate patterns of container use and quantify water turnover by water source in rural areas, although this is likely to vary depending on local cultural and socioeconomic conditions.

Although in **Chapter 3** we showed *Ae. aegypti* infestation on vehicles, several questions remain. For example, it is unclear as to how sustainable/ resilient boat-dwelling mosquito populations are. Longitudinal collections of adult mosquitoes could be conducted as a boat is travelling, although immature collections will not be possible due to cargo filling up the cargo holds during boat travel.

In **Chapter 5**, we explored the genetic relatedness of mosquito subpopulations from Iquitos and five other towns. While our results clearly demonstrated that human transit (specifically river boats) are responsible for *Ae. aegypti* long distance dispersal, *Ae. aegypti* population dynamics in the Peruvian Amazon could be better understood by conducting population genetic analysis on more contemporary samples. A comparison of mosquitoes collected from highway communities with those from riverine communities would shed light on gene flow (and therefore the degree of propagule pressure) from Iquitos to those areas. In addition, collections could be made from Iquitos, different vehicles (river boats and buses), and surrounding towns. This would provide a more detailed portrait of the invasion process.

6.3 Theoretical Contributions to Invasion Ecology

In this dissertation, I have used an invasion ecology lens to further our knowledge of *Ae. aegypti* invasion dynamics. Also worthy of consideration is what conclusions might be drawn about invasion ecology, using *Ae. aegypti* as a model system.

First, in **Chapter 2**, I considered the propagule pressure hypothesis by conducting a series of univariable and multivariable logistic regression models for *Ae. aegypti* presence/ absence. The independent variables for this model were related to both habitat suitability (i.e.- number of available oviposition sites) and propagule pressure (i.e.- transportation and connectivity information). The best multivariable model included the independent variables Log (human population) and the distance (km) from Iquitos. Interestingly, these variables are related to both habitat suitability and propagule pressure. In other words, settlements with large human populations are also highly connected to other settlements, and these communities will also have a high number of available oviposition sites. From this finding, I propose that the propagule pressure hypothesis may not be relevant for invasive species highly adapted to human environments, as variables related to habitat suitability and connectivity will always be correlated in the context of human systems.

Results from **Chapter 3** and **Chapter 4** showed highly aggregated distributions of *Ae. aegypti* infestation of vehicles, leading us to propose the concept of "super-transporters." In other words, a very small proportion of boats produces (and perhaps spreads) the overwhelming majority of mosquitoes. Identifying super-transporters of mosquitoes could help with targeted control of urban mosquitoes and other invasive species. Further, in **Chapter 4,** we added the observation that a small proportion of oviposition sites within boats is responsible for the majority of mosquito production.

In our population genetic analysis (**Chapter 5**) we proposed a "Propagule Pressure Index" combining transportation data with data on vehicle infestation to evaluate genetic isolation by distance. The Propagule Pressure Index represents an innovative way to incorporate invasion ecology concepts (the frequency and intensity of new introductions) together with population genetics, and could be a useful tool in other anthropophilic organisms.

6.3 Figures

Figure 6.1. Summary of Findings and Directions for Future Research.

	Native range	Uptake from native range	Transport on vehicle	Release into new environment	Establishment, Spread
Answered Here	• Port mosquitoes are more likely to spread.		• Different vehicle types carry *aegypti*, some more than others. • A small proportion of vehicles has the most mosquitoes. • *Ae. aegypti* oviposition occurs regularly on boats. • Limited seasonal variation in *Ae. aegypti* abundance on vehicles.		• Towns with large human populations and that are closer to cities are more likely to have *Ae. aegypti*. • Dispersal along rivers is farther.
Remaining Knowledge Gaps	• Spatial and temporal changes in population abundance • Effect of mosquito population on dengue transmission	• How/ why/ when mosquitoes initially invade boats • Importance of different life stages	• Optimal control strategies on vehicles • Population dynamics of mosquitoes on boats • Epidemiological significance of boats • Infestation of vehicles elsewhere in the world. • Infestation of vehicles for different invasive organisms.	• How/ why/ when mosquitoes exit the boat • Importance of different life stages	• Water types and mosquito oviposition preference