

**Distributions, Movements, and Arboviral Dynamics of
Invasive *Aedes* spp. Mosquitoes in US Gulf States**

By

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Abstract

The Gulf Coast of the United States is a region at high risk for the introduction and establishment of several mosquito-borne pathogens of high concern, including Zika virus, dengue virus and chikungunya virus. The Gulf Coast region plays host to both invasive mosquito species known to transmit these viruses, *Aedes aegypti* and *Aedes albopictus*, however there are critical gaps in our understanding of these species' ecologies in this region. Understanding the distributions and movements of these mosquito vector species is critical to understanding the dynamics of potential outbreaks associated viruses, and to applying effective public health and vector control strategies for disease prevention. Here we present data on the distributions of invasive *Aedes* species in the understudied US Gulf State of Alabama, which suggest broad distributions of *Ae. albopictus* throughout the state as well as a new invasion by *Aedes japonicus japonicus*. We also describe potential movement into and between US Gulf Ports by *Ae. aegypti* and *Ae. albopictus* through maritime trading routes and highlight the risk to ports such as Houston, TX, which have particularly high connectivity to outside ports where these species are abundant. Taken together, these works provide information on the risks posed by these mosquito vector species to public health in the US Gulf Coast and provide suggestions for future research and for targeted public health and vector control strategies in this region.

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Preface:

Despite public health efforts throughout much of the last century, mosquito-borne pathogens continue to negatively impact human health and economic development around the world, and many have been on the rise for decades (Gubler 1998, Gubler 2002, Gould et al. 2017, Rosenberg et al. 2018). Mosquito-borne viruses, which are often categorized in the broader group of arthropod-borne viruses (arboviruses), represent the most diverse clade of mosquito-borne pathogens as well as the most common cause of mosquito-borne infections of humans worldwide. Mosquitoes are known to transmit at least 144 viruses representing four families: *Flavaviridae*, *Togaviridae*, *Peribunyaviridae* and *Phenuiviridae* (the latter both formerly part of *Bunyaviridae*; Weaver and Reisen 2010, Grubaugh et al. 2013). Of these 144 mosquito-borne viruses, 26 viruses affect humans and nearly all of them are considered either emerging or re-emerging threats to public health (Cleton et al. 2012, Sigfrid et al. 2018). Arboviruses such as yellow fever virus (YFV; *Flavaviridae*) and Dengue fever virus (DENV; *Flavaviridae*) are reemerging as common infections after decades in decline (Bhatt et al. 2013, Grobbelaar et al. 2016, Barrett 2018, Espinal et al. 2019). Emerging arboviruses such as Keystone virus (KEYV; *Peribunyaviridae*), Mayaro virus (MAYV; *Togaviridae*) and Usutu virus (USUV; *Flavaviridae*) have caused significant concerns in localized regions where transmission of these viruses has been observed (Haddow et al. 2016, Esposito et al. 2017, Clé et al. 2019, Lednicky et al. 2019, Roesch et al. 2019). While transmission of most of these emerging arboviruses remains confined to localized ranges in the tropics, MAYV and USUV, in particular, are considered to have potential for global transmission due to their growing incidence and the ubiquity of their mosquito vectors (Esposito et al. 2017, Gould et al. 2017, Roesch et al. 2019). Other emerging arboviruses, such as West Nile virus (WNV; *Flavaviridae*), Chikungunya virus (CHIKV; *Togaviridae*) and Zika virus (ZIKV; *Flavaviridae*), have already achieved broad distributions,

causing epidemics across the globe (Charrel et al. 2007, Weaver and Reisen 2010, Bala Murugan and Sathishkumar 2016, Fauci and Morens 2016, Baud et al. 2017).

Of the 26 currently known mosquito-borne arboviruses of medical importance, four (DENV, CHIKV and YFV and ZIKV) are widely considered to pose the greatest threat (past and present) to global health (Wilder-Smith et al. 2017, Kraemer et al. 2019, Souza-Neto et al. 2019, Girard et al. 2020). Dengue fever virus represents the single greatest cause of arboviral infection in the world. Dengue fever virus is estimated to infect ~390 million people a year, a number which has risen 30-fold in the past 30 years, while more 1 billion people are estimated to be at risk from DENV each year (Bhatt et al. 2013). While the current global burden of CHIKV remains unclear due to inadequate surveillance and underdiagnosis, the virus has been responsible for multiple explosive outbreaks among naïve populations in Asia, the Indian Ocean, Europe, and the Americas (Staples et al. 2009, Amraoui and Failloux 2016, Bala Murugan and Sathishkumar 2016, Yactayo et al. 2016). Zika virus, meanwhile, has remained relatively unknown and understudied from its discovery in 1947 (Simpson 1964) until 2007 until an outbreak on Yap Island in the Federated States of Micronesia resulted in the infection of roughly 73% of the population (Lanciotti et al. 2008, Duffy et al. 2009). Following this Yap Island outbreak, another outbreak of ZIKV in French Polynesia beginning in 2013 initiated the spread of the virus to the Americas, resulting in the 2015-2016 ZIKV epidemic which infected millions of people in more than 50 countries (Cao-Lormeau et al. 2014, Fauci and Morens 2016, Paixão et al. 2016, Baud et al. 2017, Gubler et al. 2017, Silva et al. 2018). Yellow fever virus, once the scourge of Africa and much of the Americas (Downs 1982, Crosby 2007), has also caused explosive outbreaks in Angola (2015-2016), the DRC (2016), Nigeria (2017), and Brazil (2016-2018; Barrett 2018).

While many arboviral infections, including DENV, CHIKV and ZIKV, may be asymptomatic or cause relatively minor febrile disease, patients infected with DENV and YFV may develop serious, acute and life-threatening illness (Katzelnick et al. 2017, Barrett 2018), while DENV, CHIKV and ZIKV infections are all associated with potentially devastating long-term sequelae (Solomon et al. 2000, García et al. 2011, Calvet et al. 2016, Smith and Mackenzie 2016, van Aalst et al. 2017). Infection with YFV has the highest mortality rate (~50% in severe cases), but is also the most easily preventable of these arboviruses since there exists a highly effective vaccine for the disease (Barrett 2018). More than 80% of DENV infections are completely asymptomatic, but around 2% of infections lead to a more severe form of disease known as dengue hemorrhagic fever (DHF) which can be fatal (Katzelnick et al. 2017). There is also evidence of long-term neurological and autoimmune-related sequelae to DENV infection that may persist for up to 2 years post-infection (Solomon et al. 2000, García et al. 2011). Long-term sequelae of Chikungunya, namely persistent arthralgia/arthritis, alopecia, and depression also may last years (Goupil and Mores 2016, van Aalst et al. 2017). ZIKV has gained perhaps the most attention of any of these arboviruses due to its association with microcephaly in affected infants (Calvet et al. 2016, Mlakar et al. 2016) as well as the strong association between ZIKV infection and Guillaine-Barré syndrome, a rare form of autoimmune-induced flaccid paralysis that can cause lifelong disability (Smith and Mackenzie 2016).

Several anthropogenic factors have contributed to the resurgence and spread of mosquito-borne pathogens in recent decades. Insecticide resistance, a legacy of 20th century vector control efforts, has complicated vector control strategies for many mosquito vectors (Brown 1958, Hemingway and Ranson 2000, Ranson and Lissenden 2016, Moyes et al. 2017). A warming climate and shifting patterns of precipitation have led to changes in geographic and temporal

habitat favorability for many mosquito species (Gould and Higgs 2009, Tabachnick 2016, Gould et al. 2017). Rapid urbanization, especially in the global south, has also created ideal conditions for the survival and proliferation of anthropophilic vector mosquitoes such as *Aedes* (*Stegomyia*) *aegypti* (L.) and *Aedes* (*Stegomyia*) *albopictus* (Skuse; Weaver 2013, Zahouli et al. 2017, Wilke et al. 2019). While much of the world has grown increasingly suitable for vector species habitation, increasing globalization of human trade and travel have provided a vehicle for the movement of these mosquitoes, as well as their associated pathogens, into novel habitats (Lounibos 2002, Tatem, Hay, et al. 2006, Tatem, Rogers, et al. 2006, Tatem et al. 2012, Braack et al. 2018, Pliego Pliego et al. 2018, Kraemer et al. 2019).

Shifts in climate and habitat, combined with introductions of invasive species, have led to widespread replacement of native mosquito species with invasive anthropophilic vector species such as *Ae. aegypti*, *Ae. albopictus* and *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald), which has allowed for the establishment of arboviruses associated with these vectors in novel regions (Lounibos 2002, Juliano and Lounibos 2005, Medlock et al. 2012, Bonizzoni et al. 2013, Schaffner et al. 2013, Freed et al. 2014, Kampen and Werner 2014, Akiner et al. 2016, Kraemer et al. 2019). While *Ae. japonicus* has not been implicated in any major arboviral outbreaks, some populations have demonstrated anthropophagic behavior as well as laboratory competence to transmit a number of arboviruses, including DENV and CHIKV (Sardelis and Turell 2001, Molaie et al. 2009, Schaffner et al. 2011). Both *Ae. aegypti* and *Ae. albopictus* are considered important vectors of arboviruses such as DENV, CHIKV, YFV and ZIKV (Gratz 2004, Bonizzoni et al. 2013, Kraemer et al. 2019, McKenzie, Wilson, et al. 2019, Souza-Neto et al. 2019). *Ae. aegypti* is widely considered to be the primary vector of all four viruses in most parts of the world (Souza-Neto et al. 2019) and *Ae. albopictus* has been implicated in a number of

outbreaks of these viruses outside of its native Asian range (Vazeille et al. 2007, Gould et al. 2010, Zhao et al. 2016, Giron et al. 2019).

Aedes aegypti, commonly known as the yellow fever mosquito, is perhaps the most infamous mosquito species in existence due to its long history of close association with humans and its role as the primary vector of many pathogens of high concern to the global health community. *Aedes aegypti* was one of the first mosquito species to become globally invasive, being transported from Africa around the world via the so-called “triangular trade” of slaves and goods by colonial nations early as the 16th and 17th centuries (Powell et al. 2013). While remnant populations of native West-African subspecies *Ae. aegypti formosus* breed in tree-holes and tend towards zoophagy (Lounibos 1981), the more broadly distributed and invasive *Aedes aegypti aegypti* breeds preferentially in man-made containers such as clay pots, discarded tires and plastic containers, and is highly anthropophilic (Christophers 1960, Nelson 1986, Powell et al. 2013). *Aedes aegypti* is almost exclusively found in close proximity to humans, so much so that it is commonly referred to as a “domesticated species,” and *Ae. aegypti* distribution and abundance are highly correlated with urban development (Christophers 1960, Nelson 1986, Simard et al. 2005, Zahouli et al. 2017, Abílio et al. 2018). *Aedes aegypti* distributions are further limited by temperature, with rates of reproduction and adult survival among *Ae. aegypti* populations dropping precipitously at temperatures below 13-16°C (Rueda et al. 1990, Yang et al. 2009, Brady et al. 2013, Reinhold et al. 2018, Tsai et al. 2018).

Aedes albopictus began its global invasion far more recently than its African cousin. Beginning in the 1980s, *Ae. albopictus* was transported from its native Southeast Asia by the international trades in lucky bamboo (*Dracaena sanderiana*) and used tires (Pratt et al. 1946, Reiter and Sprenger 1987a, Hofhuis et al. 2009, Medlock et al. 2012, Pliego Pliego et al. 2018).

In the following decades, *Ae. albopictus* has come to occupy a near-global distribution (Bonizzoni et al. 2013, Kraemer et al. 2015, 2019). *Aedes albopictus* can withstand far lower temperatures than *Ae. aegypti*, breeding continuously in temperatures as cold as -5°C and producing eggs capable of entering a state of dormancy for up to six months during times of temperature- or desiccative-stress (Estrada-Franco and Craig 1995, Benedict et al. 2007, Paupy et al. 2009, Bonizzoni et al. 2013). While most populations of *Ae. albopictus* tend towards anthropophagy and thus favor more urban and suburban environments, *Ae. albopictus* populations tend to exhibit a high level of ecological plasticity, feeding and breeding opportunistically (Estrada-Franco and Craig 1995, Richards et al. 2006, Benedict et al. 2007, Delatte et al. 2008, Paupy et al. 2009, Kamgang et al. 2012, Bonizzoni et al. 2013, Faraji et al. 2014). Because of their ecological plasticity, *Ae. albopictus* may be found in rural environments as well as areas of urban development (Simard et al. 2005, Abílio et al. 2018, Kamgang et al. 2018).

Like *Ae. albopictus*, *Ae. japonicus* left its native Asian distributions relatively recently, with the first field samples of the species collected in North America in 1998, and in Europe in 2000 (Sardelis and Turell 2001, Fonseca et al. 2010, Schaffner et al. 2013, Kampen and Werner 2014). Like *Ae. albopictus*, *Ae. japonicus* was likely transported via the maritime trade in lucky bamboo and used tires (Medlock et al. 2012, Schaffner et al. 2013, Kampen and Werner 2014, Kaufman and Fonseca 2014). Conflicting reports of *Ae. japonicus*' ecology exist both within and outside of its native range. Most sources claim that within Japan *Ae. japonicus* preferentially breeds in rock pools (Tanaka et al. 1979, Kampen and Werner 2014), while in Europe and North America it has adapted to a wider variety of breeding habitats, including the types of containers used preferentially by *Ae. albopictus* and *Ae. aegypti* (Andreadis et al. 2001, Schaffner et al.

2009, Kaufman and Fonseca 2014, Krebs et al. 2014). The blood-meal preferences of *Ae. japonicus* seem to vary regionally, with some populations in Japan and the northeastern United States preferentially feeding on non-human, mammalian hosts (LaCasse and Yamaguti 1948, Apperson et al. 2004), while other sources from neighboring regions suggest aggressive behavior towards humans, and even moderate levels of anthropophagy (Knight 1968, Iriarte et al. 1991, Molaei et al. 2009). *Aedes japonicus* seems to be capable of withstanding cold temperatures, with larvae found in northeastern Japan and the southern Appalachians at altitudes as high as 1,500 m, where temperatures can fall as low as -15°C (Tanaka et al. 1979, Bevins 2007, Kampen and Werner 2014). *Aedes japonicus*' upper thermal limits are less clear, but since its arrival in the United States in 1998 it has slowly moved southward into warmer climates, arriving as far south as Mississippi and the northeast corner of Alabama (Qualls and Mullen 2006, Goddard et al. 2017).

Because *Ae. aegypti*, *Ae. albopictus* and *Ae. japonicus* occupy broad distributions and have relatively short flight ranges, and thus are limited in terms of self-powered dispersal (Guerra et al. 2014), there is often important variation between populations in terms of ecologies, vector competence and resistance to insecticides (Lambrechts et al. 2009, Vontas et al. 2012, Fansiri et al. 2013, Manni et al. 2017). While the distributions, ecologies, and life histories of *Ae. aegypti*, *Ae. albopictus* and *Ae. japonicus* are well studied and well documented in many parts of these species' ranges, critical gaps exist in our understanding of these species in regions where they have not been as extensively studied. This thesis aims to fill in some of the gaps in our understanding of these invasive species' distributions, ecologies and movements, as well as associated pathogen dynamics, in a region at high risk for the establishment of arboviruses such as ZIKV, DENV, and CHIKV: the US Gulf Coast.

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Chapter 1:

Distributions and Pathogen Dynamics of the Invasive Mosquito (Diptera: Culicidae) Vectors *Aedes aegypti*, *Aedes albopictus*, and *Aedes japonicus japonicus* in Alabama

Introduction

Understanding the distributions and ecology of mosquito vectors of disease is crucial to understanding disease dynamics, as well as to developing vector control strategies and other public health responses to outbreak of disease (Messina et al. 2015, Fernandes et al. 2018, Franklino et al. 2019, Petersen et al. 2019, Lwande et al. 2020). Vector surveillance, when timely and accompanied by tailored vector control strategies, has proven effective in reducing incidence of mosquito borne disease (Ooi et al. 2006, Scott and Morrison 2010, Eskildsen et al. 2020). Prediction of vector distributions and populations through the use of remote sensing, environmental data and geographic information systems (GIS) is likely to play a major role in vector surveillance moving forward (Reiner et al. 2013). However, these geospatial models rely on field observations of mosquito distributions for their creation and calibration, observations which can be scarce in areas with little funding for public health departments to carry out surveillance (Gubler 1998, Reiner et al. 2013, Kraemer et al. 2015). Furthermore, invasions by insect vector species via human trade and travel routes can cause rapid shifts in vector species populations, with some invasive species expanding their ranges and others being outcompeted (Hosono 1998, Lounibos 2002, Juliano and Lounibos 2005), interactions which can be difficult to integrate into spatial models (Eisen and Moore 2013, Kraemer et al. 2015). Field surveillance can also include pathogen surveillance, providing early warning of potential outbreaks, which can be especially critical with often asymptomatic infections such as those caused by many arboviruses (Petersen et al. 2019, Eskildsen et al. 2020).

The US state of Alabama provides a perfect example of the need for improved surveillance and understanding of vector ecology. Following the introduction of *Ae. albopictus* to the southeastern US in 1985, *Aedes aegypti* was thought to have been extirpated from Alabama through competition for resources between larval *Ae. albopictus* and *Ae. aegypti*, as well as through adult satyrization, by which process *Ae. albopictus* males would mate with and sterilize *Ae. aegypti* females (Hobbs et al. 1991, Qualls and Mullen 2006, Bargielowski and Lounibos 2016). However, the rediscovery of *Ae. aegypti* populations in Mobile, AL and in Huntsville, AL in 2017 pointed to the existence of either remnant or newly invading populations (Hahn et al., 2017, Zohdy et al., 2018). Additionally, little data has been reported on the distributions and ecology of *Ae. albopictus*, or the newly invading vector species *Ae. japonicus* within Alabama. What data have been reported have focused entirely on larval distribution and abundance (Qualls and Mullen 2006, Kraemer et al. 2015). Almost no work has been done to assess arboviral presence among mosquito populations in the state, despite the proximity of states with autochthonous transmission of ZIKV, DENV, and CHIKV (Graham et al. 2011, Adalja et al. 2012, Kendrick et al. 2014, Dinh et al. 2016, Likos 2016) as well as models showing high risk for transmission of various *Aedes*-borne arboviruses in the state (Leta et al. 2018). Filling this gap in surveillance will allow the creation of models and predictions of mosquito vector movement, ecology and arboviral disease dynamics, helping public health agencies to create viable vector control strategies (Eisen and Moore 2013, Kraemer et al. 2015, Zohdy et al. 2018).

The primary goal of this study was to address gaps in surveillance regarding invasive *Aedes* spp. mosquitoes and their associated arboviruses in the state of Alabama. The objectives of this study were to: 1) determine the distributions of invasive *Aedes* spp. mosquitoes within the state of Alabama; 2) look for evidence of arboviruses of public health concern, and with potential

for establishment in Alabama, namely ZIKV, DENV and CHIKV, within *Aedes* spp. mosquito populations in the state; and, 3) identify the underlying ecological drivers of invasive *Aedes* spp. mosquito distributions around the state. Based on previous observations of mosquitoes within the state, we hypothesized that *Ae. aegypti* and *Ae. albopictus* would both occupy statewide distributions, while *Ae. japonicus* would likely be found more commonly in the northeastern part of the state. We also hypothesized that ZIKV, DENV, and CHIKV would all be present within the state, but concentrated in the southern portion, where climate and proximity to ports of entry would facilitate establishment. Finally, we hypothesized that *Ae. aegypti*'s distribution within the state would be primarily driven by human development, while the distribution of *Ae. albopictus* would be driven by availability of containers for breeding and *Ae. japonicus* by temperature.

Methods

Specimen Collection

Across the 67 counties of Alabama I sampled three to four sites per county for a total of 203 sites. Sites were pre-selected using Google Maps to locate tire shops and other sites (junkyards, etc.) where we were likely to find an abundance of the water-holding containers necessary for *Aedes* mosquito reproduction. We focused on identifying tire shops because several *Aedes* species important for public health preferentially oviposit in stagnant water in tires (Reiter and Sprenger 1987a, Honório et al. 2006, Yee 2008, Burkett-Cadena 2013). In counties without many tire shops, other sites with suitable breeding habitat (cemeteries, abandoned buildings) were identified. Sites were selected to provide optimal geographic and land-use

heterogeneity within each county, meaning when possible sites were distributed evenly throughout the county and within areas of both urban and rural land cover.

Sites consisted of a 50-m radius around each selected sampling location. Each site was visited at least twice between May and August 2018 to account for incomplete detection (MacKenzie et al. 2003). Total number of open containers (tires, flower pots, trash, etc.), as well as the container index (CI), or the percentage of open containers infested with mosquito larvae of any species, was recorded at each location (WHO Vector Surveillance 2019). Adult mosquitoes were captured by aspirating each site for 20 min using Prokopack (John W. Hock Company, Gainesville, FL) backpack aspirators (Vazquez-Prokopec et al. 2009). Open containers, vegetation and other likely mosquito landing sites were disturbed during aspiration to ensure maximum capture rates.

Specimen Identification

Captured adult mosquitoes were transported from sites to a laboratory or mobile laboratory location within Prokopack aspirator collection cups (John W. Hock Company, Gainesville, FL), where they were euthanized by enclosing them with a preparation of cotton balls soaked in acetone. Adults were then transferred to a sterile petri dish and examined under 10x magnification using a Boreal2 stereo microscope (VWR International, Radnor, PA) and identified morphologically to species using a dichotomous key (Burkett-Cadena 2013).

For a subset of adult *Aedes* spp. mosquitoes (n = 16), species identification was confirmed molecularly. DNA was extracted with the EZNA tissue kit (Omega Bio-tek, Norcross, GA) from specimens identified morphologically as either *Ae. aegypti*, *Ae. albopictus*, or *Ae.*

japonicus. Extracted DNA was used as PCR template to amplify the mitochondrial gene cytochrome c oxidase subunit I (COI) using the primers and reaction conditions of Kumar et al. (Kumar et al. 2007). The PCR primer sequences were: Forward-5-GGATTTGGAAATTGATTAGTTCCTT-3, Reverse 5-AAAAATTTTAATTCCAGTTGGAACAGC-3. Reactions were confirmed by gel electrophoresis, and amplicons were ligated into a plasmid and cloned in *E. coli* using the CloneJet PCR cloning kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Plasmid DNA was purified using the GeneJet plasmid miniprep kit (Thermo Fisher Scientific), quantified by spectrophotometer and outsourced for Sanger sequencing (Eurofins Genomics). Returned sequences were searched against the NCBI Nucleotide collection using BLAST

Viral Detection

As *Ae. albopictus* was the only mosquito collected which has demonstrated competence for transmitted ZIKV, DENV, and CHIKV, all adult female (1,875) *Ae. albopictus* were assayed to determine infection status. Even females that were not visibly bloodfed were included to avoid missing cryptically bloodfed individuals. Individuals were dissected using forceps and scalpels to isolate the salivary gland between the thorax and the head. All dissections took place on dry ice to prevent freeze-thaw and deterioration of viral RNA. Salivary glands were then pooled by species and site, with up to 5 salivary glands per pool, depending on the number of *Ae. albopictus* captured at each site. These pools were then returned to -20°C storage.

Viral RNA was extracted from mosquito pools using Qiagen RNeasy Mini Kits (Qiagen Corp., Hilden, Germany). Extracted viral RNA was then assayed for ZIKV, DENV, and CHIKV

presence using a ZDC Multiplex RT-PCR Assay Kit (Bio-Rad Laboratories Inc., Hercules, California, USA) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, California, USA). These kits are highly sensitive to ZIKV, DENV (all four serotypes), and CHIKV, and also highly specific to these viruses (Thompson and Islam 2018). All assays were performed in duplicate to control for testing validity. Pools were potential positives if they amplified before the 30th amplification cycle (Paiva et al. 2017). Amplicons from pools for which at least one replicate tested positive were cloned into TA plasmid vectors for confirmation via Sanger sequencing.

Environmental Characteristics Associated with Invasive *Aedes* spp. Distributions

Following previous studies which used “expert knowledge-based models” to model distributions of *Aedes* spp. mosquitoes in other regions (Fischer et al. 2011, Koch et al. 2016), we included mean annual temperature and mean annual precipitation for the years 1970-2000, were downloaded from WorldClim (<https://www.worldclim.org/>) at a resolution of 2.5 arc minutes. Mean annual precipitation affects the ability of mosquitoes to breed, as they rely on water for oviposition and larval development, while low mean monthly temperatures can affect species’ ability to overwinter (Mitchell 1995). Mean annual temperatures are highly correlated with mean monthly temperatures, and have proven to be better at predicting *Aedes* species’ distributions (Fischer et al. 2011, Koch et al. 2016). Because *Ae. aegypti* is considered a purely urban species, while *Ae. albopictus* and *Ae. japonicus* are often thought to prefer peri-urban or sylvatic environments, we also used a remotely-sensed development index to account for the effects of urban development on species presence (Weaver 2013, Zahouli et al. 2017, Wilke et al. 2019). Land cover data with a resolution of 30m was downloaded from the 2016 National Land Cover Database (NLCD; <https://www.mrlc.gov/national-land-cover-database-nlcd-2016>). Land

cover from the dataset was divided into developed and undeveloped and the percent of developed land within a 400 m radius (commonly considered to be the average habitat range for *Aedes* spp.) of each site was calculated in QGIS (QGIS Development Team 2020). Since we expected to see an effect of available breeding habitat on distribution and abundance, both the total number of containers present and average CI at each site were used as variables in this analysis. A total of 198 sites were used for this analysis; 5 sites were dropped due to difficulty extracting environmental variables for these sites.

We tested for collinearity among variables by producing a correlation matrix, as well as by using a variance inflation factor (VIF) analysis, but found no evidence of collinearity among any variables ($VIF < 2.5$). Environmental data were analyzed with two fixed-effects logistic regression models in order to determine the effects of bioclimatic and habitat variables on the distributions of *Ae. albopictus* and *Ae. japonicus*, as well as two fixed-effects general linear models to determine the effects of variables on average abundance *Ae. albopictus* and of *Ae. japonicus* at each site. All analyses were conducted using the program R (R Core Team 2019).

Results

Aedes spp. Populations and Distributions

We captured a total of 4,595 adult mosquitoes: 3,408 *Ae. albopictus* (74.2%), 62 *Ae. japonicus* (1.3%), 25 *Aedes* spp. (<1%), 7 *Aedes (Protomacleaya) triseriatus* (Say) (<1%), 6 *Aedes (Ochlerotatus) atropalpus* (Coquillett) (<1%), 1 *Aedes (Ochlerotatus) atlanticus* (Dyar and Knab) (<1%), 997 *Culex* spp. (21.7%), 24 *Anopheles* spp. (<1%), 2 *Psorophora* spp. (<1%), and 1 *Orthopodomyia signifera* (Coquillett) (<1%). A further 11 adults were suspected to be *Ae.*

aegypti could not be confirmed by morphology but were identified as different species by molecular methods. Sequencing of the mitochondrial gene COI revealed a single haplotype for two *Ae. albopictus* specimens (GenBank accession numbers: MK372913, MK372914; barcode index number: BOLD:AAA5870) and three haplotypes for *Ae. japonicus* (GenBank accession numbers: MK372910, MK372911, MK372912; barcode index number: BOLD:AAC5210) among the samples collected. In all, *Ae. albopictus* adults were collected at 173 sites representing 65 counties (Figure 1) and *Ae. japonicus* adults at 36 sites representing 30 counties (Figure 1). Of the *Ae. albopictus* captured, 55% were female, 34% were male and 11% were not sexed. Of the *Ae. japonicus* captured, 42% were female, 24% male and 34% were not sexed. Investigation of larval breeding habitat revealed a mean of 214.8 containers (± 18.8 ; \pm SE) at each collection site (Figure 2a). Mean CI, or the proportion of containers at each site that held larvae, was high in counties across the state, ranging 5%-74% with an overall mean site CI of 31.1% (± 1.6 %; \pm SE; Figure 2b).

Viral Detection

A total of 8 samples, representing 8 different sites in 7 counties (Table 1) tested positive for DENV, however only one replicate of each assay tested positive. We also detected CHIKV in 2 samples from 2 separate counties and ZIKV in 1 sample from a single county (Table 1 and Figure 3), although likewise we failed to detect virus in both replicates of these samples. An initial attempt to clone the amplicons from these positive samples into TA plasmid vectors in order to enable Sanger sequencing confirmation, but we were unable to obtain any viral RNA from these amplicons.

Environmental Characteristics Associated with Invasive *Aedes* spp. Distributions

We found that the only number of containers present and average CI at each site were significant ($P < 0.05$) related to the distribution and abundance of *Ae. albopictus* and *Ae. japonicus*. We observed 1.01 times as many total containers (0.01-2.02, 95% CL; $P = 0.003$) and 1.05 times as high of a container index (0.02-2.12, 95% CL; $P < 0.001$) at sites occupied by *Ae. albopictus*. We also observed 1.04 times as high of a container index at sites occupied by *Ae. japonicus* (0.02-2.09, 95% CL; $P < 0.001$), but we observed no significant effect of total containers per site on *Ae. japonicus* presence ($P = 0.3$). For each 1 container increase in containers per site, we observed an increase of 0.02 (± 0.01 ; 95% CI) *Ae. albopictus* per site ($P < 0.001$), while for each 1% increase in CI, we observed an increase of 0.13 (± 0.07 ; 95% CI) *Ae. albopictus* per site ($r^2 = 0.18$; $P < 0.001$). We found no significant effects of any environmental variables on *Ae. japonicus* abundance ($r^2 = -0.01$; $P = 0.56$) and no significant effect of mean annual temperature, mean annual precipitation or developed landcover on distribution or abundance of either *Ae. albopictus* or *Ae. japonicus*.

Discussion

Aedes spp. Populations and Distributions

Both mean statewide CI and mean individual CIs for sites across the state were above the 10% acceptable limit dictated by the Alabama Department of Public Health (Figure 2b). Used tire shops are required to regularly treat containers with insecticides, thus the high CIs observed may suggest either broad resistance to insecticides (Vontas et al. 2012) or low efficacy in the way these insecticides are used. Both larvicides and adulticides require specialized training and

ideal conditions for maximum efficacy (Rose 2001), and we anecdotally observed that the majority of tire-shop owners, especially those outside of city-controlled mosquito control programs, performed their own insecticide applications. While we collected no empirical data on site microclimate and mosquito presence or abundance, we observed that poorly kept tires and other containers, especially those kept in the shade or near vegetation, tended to contain larvae regardless of whether property owners had recently treated these containers. Sites with the lowest container indices tended to be sites within the city-limits of a city with a mosquito-control program and sites where tires and other containers were regularly moved to prevent accumulation of water and then housed in open sunlight, away from vegetation.

The ubiquity of *Ae. albopictus* (Figure 1a) and the seeming absence of *Ae. aegypti* throughout the state are in line with previous larval studies in Alabama (Qualls and Mullen 2006) and surrounding states (Goddard et al. 2017). However, the widespread occurrence of *Ae. japonicus* throughout the state (Figure 1) suggests considerable southward expansion by *Ae. japonicus* since it was first reported in a single county in 2005 (Qualls and Mullen 2006). This southward expansion seems to have occurred relatively rapidly, despite predictions that the thermal preferences of *Ae. japonicus* would slow and eventually halt expansion into the US South (Rochlin et al. 2013). While early studies of the ecology of North American *Ae. japonicus*, including studies of populations in the Appalachian region, suggested a preference for cooler climates, high elevations and heavily forested areas (Gray et al. 2005, Bevins 2007, Grim et al. 2007), there is evidence that it is adapting to warmer climates and more urban settings throughout its North American range (Larish and Savage 2005, Kaufman and Fonseca 2014).

The prevalence of adult *Ae. albopictus* and *Ae. japonicus* throughout Alabama (Figure 1) suggests that more research should be conducted on roles both species may play as arboviral vectors in the Southeastern United States. Both species are larval competitors and may eradicate native mosquito species (Benedict et al. 2007, Paupy et al. 2009, Kampen and Werner 2014, Kaufman and Fonseca 2014). Both species are also ecologically plastic in their selection of oviposition sites and feeding habits, making them potential bridge vectors between wildlife and humans (Paupy et al. 2009, Kampen and Werner 2014). *Aedes albopictus* is a competent vector of ZIKV, Ross River virus (RRV), West Nile virus (WNV), and yellow fever virus (YFV) and has been implicated in outbreaks of DENV and CHIKV in Europe, Africa, and East Asia (Mitchell 1991, Paupy et al. 2009, Wu et al. 2010, Vega-Rua et al. 2013, McKenzie, Wilson, et al. 2019). *Aedes japonicus*, is a somewhat understudied vector, perhaps due to its relatively recent invasions of North America and Europe (Kampen and Werner 2014). However, *Ae. japonicus* has been shown to be a competent vector of WNV, Eastern equine encephalitis virus (EEEV), Japanese encephalitis virus (JEV), LaCrosse virus (LACV), St. Louis encephalitis virus (SLEV), and Rift Valley fever virus (RVFV; Sardelis and Turell 2001, Sardelis, Dohm, et al. 2002, Sardelis, Turell, et al. 2002, Turell et al. 2013). Furthermore, larval competition between these two invasive species, as well as between these and native species, may lead to higher rates of arboviral infection by increasing larval stress and thus adult susceptibility to infection (Alto et al. 2005).

Detection of *Ae. aegypti* in Mobile county on the southern edge of Alabama and Madison county on the northern edge of the state a year prior suggests a broad range of occurrence within Alabama (Hahn et al. 2017, Zohdy et al. 2018). However, the lack of detection of *Ae. aegypti* in this study suggests either low numbers of established, high-density populations within the state,

or transient populations circulated by the used tire trade. Timing also may have played a role in the failure of this study to detect *Ae. aegypti*. Recently detected populations of *Ae. aegypti* in southern Alabama demonstrated low capture rates between the months of June and August, which coincided with the highest capture rates of *Ae. albopictus* (Zohdy et al. 2018). This temporal pattern in detection of *Ae. aegypti* in Alabama suggests that during the summer months increased numbers of *Ae. albopictus* may suppress *Ae. aegypti* populations. Future work should focus on determining the effects of seasonality and climate on *Ae. aegypti* and *Ae. albopictus* competitive interactions in this part of their range.

This study represents the first major vector surveillance effort in Alabama in over a decade, and the first ever to use both adult surveillance and CI, a methodological departure from previous vector surveillance efforts in the southeastern United States, which have focused on active larval surveillance and passive trapping methods (Qualls and Mullen 2006, Foppa et al. 2007, Britch et al. 2008). We used active sampling for adults to achieve maximum surveillance coverage within a short time frame. Active backpack aspiration is a cheap, quick, and effective way to gauge presence and absence of adult mosquitoes when compared to conventional traps which may be less effective at capturing certain species (Maciel-de-Freitas et al. 2006). Biogents Sentinel and Gravid Aedes traps were not used for this study because of the lack of *Ae. aegypti* captured in these traps in a previous study in Alabama, even at locations where aspiration yielded collections of *Ae. aegypti* (Zohdy et al. 2018).

The detection of *Ae. japonicus* in a variety of habitats across the state, including further south than the species has ever been documented before, suggests a new wave of invasion by *Ae. japonicus*, potentially fueled by adaptation to a warmer climate (Kaufman and Fonseca 2014).

Dispersal and expansion by *Ae. japonicus* and *Ae. albopictus* may occur overland by car and truck (Eritja et al. 2017, Kraemer et al. 2019), or internationally via the international maritime trade in used tires (Benedict et al. 2007, Kaufman and Fonseca 2014). Expansion and dispersal by these and other invasive vector species may lead to new interactions (Alto et al. 2005), new genotypes (Fonseca et al. 2010, Medley et al. 2015), and new arboviral dynamics (Kampen and Werner 2014). Because mosquito vectors' ecologies and distributions are dynamic, future efforts to establish efficient, long-term vector surveillance in areas of risk are necessary.

Viral Surveillance

While our initial RT-qPCR results indicated positives for all three target arboviruses (DENV, CHIKV, and ZIKV) from a number of samples from across the state (Table 1 and Figure 3), our inability to duplicate these results, or to isolate amplicons for sequencing, suggests that these may have been false positives. While our failure to definitively detect these arboviruses among our samples suggests that autochthonous transmission of these viruses is not common within the state, it does not mean that autochthonous transmission does not occur. Even in areas where arboviral transmission is endemic, mosquito populations often have low infection rates, with only 1 in 1,000 individuals testing positive for infection (Gu and Novak 2004). Successful arboviral surveillance efforts targeting one or a few specific arboviruses have required years of sampling and reported sample sizes between 40,000 and 450,000 (Ochieng et al. 2013, Ndiaye et al. 2018). Given that we tested less than 2,000 individuals, our sample size may simply have been too low for effective surveillance.

Studies comparing the sensitivity of different arboviral surveillance methods concluded that human serological surveillance is a better indicator of arboviral presence than vector

surveillance, but that an integrated approach using both human serology and vector surveillance would provide the best early warning and detection system for arboviruses (Madewell et al. 2019, Eskildsen et al. 2020). New techniques such as metatranscriptomics, metabarcoding and deep sequencing also offer promising results for use in arboviral surveillance, especially as surveillance using these methods may allow for the detection of non-target viruses and may thus be more broadly useful (Coffey et al. 2014, Batovska et al. 2018, 2019). Continuing surveillance of vector species and incorporation of human serological surveillance could help to provide a complete picture of vector dynamics among the invasive *Aedes* species in the southeast and provide early warning in the event of new arbovirus introductions.

Environmental Analysis

Our chosen variables failed to accurately predict presence or abundance of either *Ae. albopictus* or *Ae. japonicus*. While we detected significant effects of average CI and total numbers of containers on presence and abundance of both *Ae. albopictus* and *Ae. japonicus*, these effects were too small to be considered biologically significant. This lack of biological significance may be due to insufficient sample size or failure to account for one or more confounding variables. Alternatively, this lack of unifying environmental predictors may be due to rapid adaptation in response to expansion and constant outside gene flow, which may cause phenotypic heterogeneity in habitat choice. *Aedes japonicus* populations in Alabama are relatively recent arrivals (Qualls and Mullen 2006), and that it is likely that some kind of adaptation to the warmer climate was necessary for their expansion into the state (Gray et al. 2005, Bevins 2007, Kaufman and Fonseca 2014). We also know that *Ae. albopictus* populations across world are in constant states of genotypic flux due to long-distance dispersal, both overland

and via maritime trade routes (Medley et al. 2015, Eritja et al. 2017, Manni et al. 2017). More work is needed to understand the influence of genotypic heterogeneity on mosquito populations around the state.

Limitations

Distribution data reported in this study is represented at the county level and does not show within-county variation of mosquito occurrence, which may convey an inflated idea of the actual distributions of these species. Also, all sampling for this study was done between May-August, 2018, in summer months when mosquitoes are most active, in order to optimize detection. Further work is necessary to determine the year-round distributions of these mosquito vectors in the Southeastern US. Because mosquito vectors' ecologies and distributions are dynamic, it will also be critical to future public health efforts to set up efficient, long-term vector surveillance in areas of risk, a task made easier by new technologies such as cell-phone wingbeat recordings, which may allow the integration of reliable, crowd-sourced mosquito surveillance data into current vector surveillance efforts (Mukundarajan et al. 2017). More work is also needed to assess the feeding behavior and vector competence of *Ae. albopictus* and *Ae. japonicus* in the Southeastern US, as these traits are known to vary geographically among mosquito populations (Estrada-Franco and Craig 1995, Lambrechts et al. 2009, Paupy et al. 2009). Finally, integrated vector and human serological surveillance may help to establish an early warning system for arboviral establishment in the state, as well as providing invaluable insight into the dynamics of currently established arboviruses (Madewell et al. 2019, Eskildsen et al. 2020).

Conclusions

The Gulf Coast of the United States has been identified as the region at greatest risk for the introduction of arboviruses such as DENV, ZIKV, and CHIKV into the United States (Zohdy et al. 2018). Warm and humid climate, urban expansion, abundance of vector species and the presence of major transportation hubs make Alabama a good model for the conditions that make the Gulf Coast ideal for arboviral establishment (Kraemer et al. 2015, Leta et al. 2018, Monaghan et al. 2018). Understanding the nature of vector distributions and disease dynamics in Alabama may be the key to preventing the establishment of arboviruses across the region.

Aedes albopictus remains the dominant container-breeding mosquito and vector species in the U.S. Gulf states. However, the recent third wave of *Aedes* invasion by *Ae. japonicus* and new detection of *Ae. aegypti* may alter vector distribution in coming years. Understanding the eco-epidemiology of these three species within the state, as well the potential for new invasions, is critical for predicting disease dynamics in the future and developing strategies to mitigate or prevent outbreaks. It is of the utmost importance to U.S. public health and national security for us to expand our vector surveillance efforts in order to prevent the emergence and establishment of mosquito-borne disease in the Gulf states.

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Figures and Tables

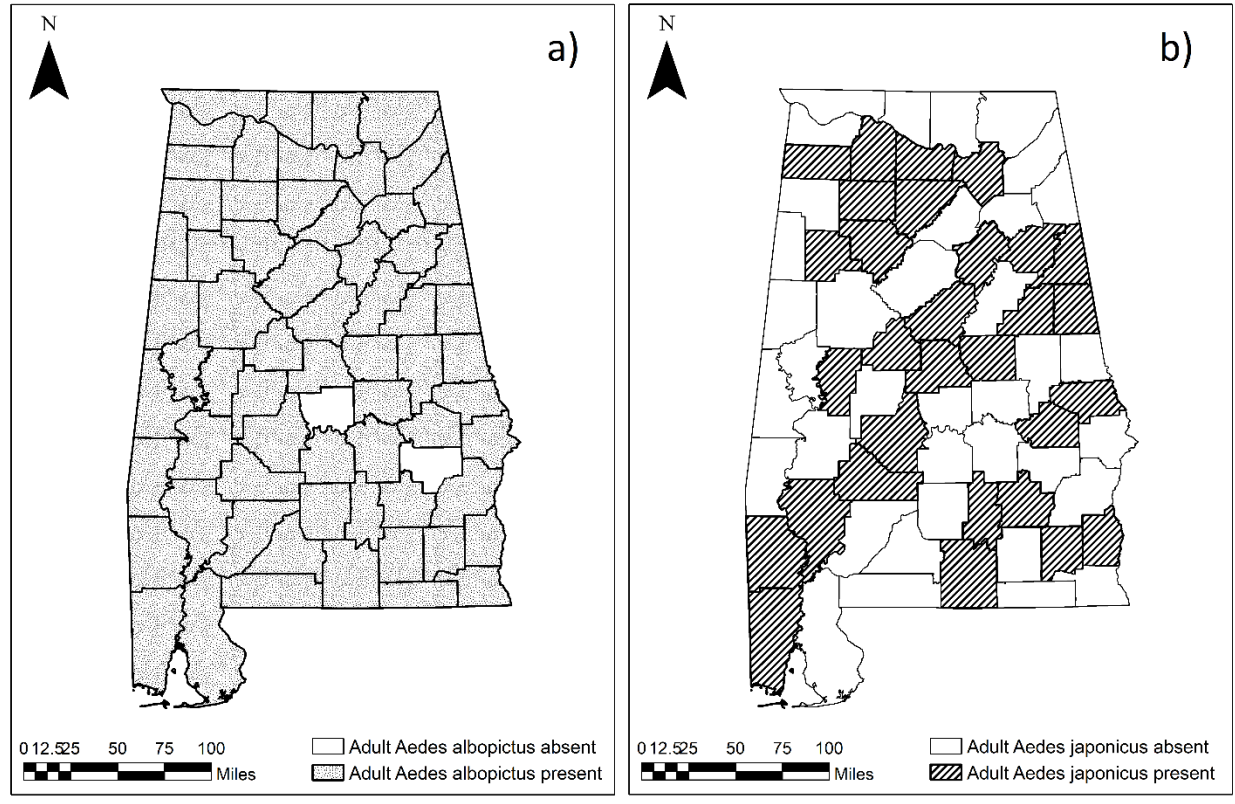


Figure 1. Maps showing presence/absence of adult *Ae. albopictus* and *Ae. japonicus* collected through backpack aspiration at the county level. A total of 3,408 *Ae. albopictus* (a) adults were captured at sites in 65/67 counties and 62 *Ae. japonicus* (b) adults were captured at sites in 30/67 counties. Sites sampled were mainly facilities with used tires and all sampling was done between May and August 2018.

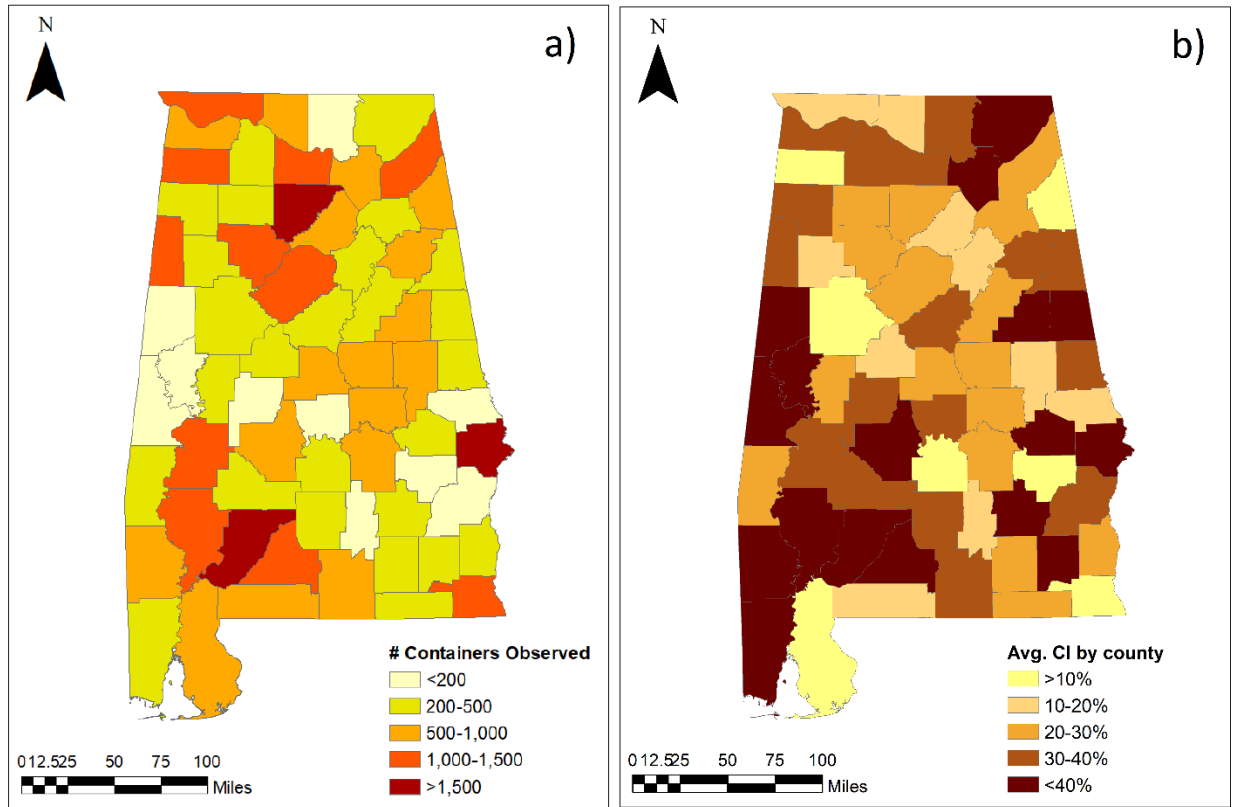


Figure 2. Containers such as tires, oil drums and other trash provide critical breeding sites for *Aedes* spp. and other container breeding mosquitoes across the state of Alabama. (a) We found an overall mean of 214.8 containers (± 18.8 ; \pm SE) at each collection site. (b) Mean CI, or the proportion of containers at each site that held larvae, was high in counties across the state, ranging 5%-74% with an overall mean site CI of 31.1% ($\pm 1.6\%$; \pm SE). Sites sampled were mainly facilities with used tires and all sampling was done between May and August 2018.

Table 1. A total of 11 mosquito pools representing 11 sites in 10 Alabama counties tested positive ($Cq < 30$) via RT-qPCR for at least 1 virus. Dengue virus was detected in samples from 7 counties, including from two separate sites in Russell County. Chikungunya virus was detected in samples from 2 counties and ZIKV in 1 sample from Morgan County.

| County | Virus Detected | Cq Value(s) (RT-qPCR) |
|------------|----------------|-----------------------|
| Conecuh | DENV | 4.97 |
| Dallas | DENV | 3.97 |
| Elmore | DENV | 3.18 |
| Mobile | DENV | 2.56 |
| Russell | DENV | 4.17, 14.86 |
| Tallapoosa | DENV | 3.68 |
| Washington | DENV | 2.05 |
| Baldwin | CHIKV | 17.74 |
| Randolph | CHIKV | 18.29 |
| Morgan | ZIKV | 13.34 |

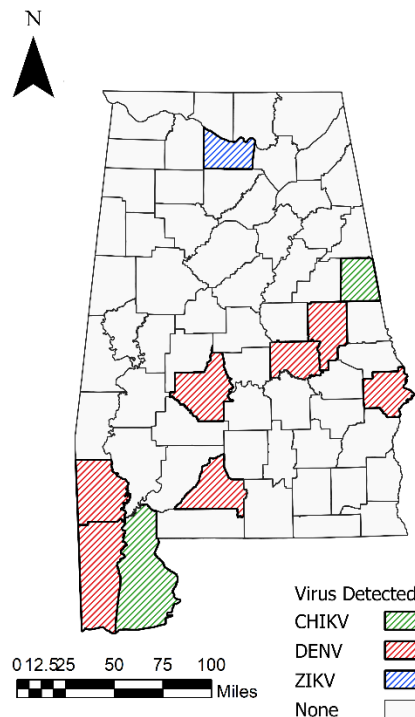


Figure 3. Initial detection of ZIKV (blue), DENV (red) and CHIKV (green) in the above counties of Alabama was made via RT-qPCR. However, these samples were not detected in duplicates and attempts to clone our amplicons for sequencing failed to recover any viral RNA, suggesting the likelihood that these detections were false positives.

Chapter 2:

Potential Routes of Reintroduction into the Gulf States, USA, by *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) via the International Maritime Shipping Network

Introduction

The globalization of trade and travel has allowed many invasive species to disperse and establish themselves in novel locations and at distances much farther than their natural dispersal abilities should allow (Hulme 2009, Banks et al. 2015). In particular the global shipping network (GSN) acts as significant pathway for the long-distance transport of invasive and other non-native organisms to novel locations (Keller et al. 2011, Banks et al. 2015). Non-native aquatic species are often transported in the ballast water, or attached to the hulls of vessels (Drake and Lodge 2004, Sylvester and MacIsaac 2010), while terrestrial species are often transported with cargo (Paini and Yemshanov 2012, Sardain et al. 2019).

International maritime trade and the GSN have been especially instrumental in the global invasions of several medically important *Aedes* spp. mosquitoes, most notably *Aedes* (*Stegomyia*) *aegypti aegypti* and *Aedes* (*Stegomyia*) *albopictus* (Skuse) (Bonizzoni et al. 2013, Powell et al. 2013, Kampen and Werner 2014). Recent studies of genetic differentiation among populations of both *Ae. aegypti* and *Ae. albopictus* indicate constant human aided, long-distance reintroduction events among established populations across the world (Brown et al. 2011, Medley et al. 2015, Manni et al. 2017, Maynard et al. 2017). Long-distance reintroduction may impact population resilience among these mosquitoes, bolstering existing populations and altering gene-flow and local genotypes (Yakob and Yan 2010, Medley et al. 2015, Fouet et al.

2017). Changes in local genotypes of these mosquitoes may also have implications for public health and vector control efforts, as localized variations in genotype effect resistance to insecticides and competence to transmit pathogens (Lambrechts et al. 2009, Vontas et al. 2012, Fansiri et al. 2013, Manni et al. 2017). These population-level effects further highlight the importance of long-distance dispersal and maritime reintroduction.

The most effective strategy in limiting the spread of invasive species, including *Ae. aegypti* and *Ae. albopictus*, along maritime trade routes is the implementation of effective biosecurity measures at points of entry such as ports (Colunga-Garcia et al. 2013, Dobson et al. 2013). Early detection and rapid response are well-established methods for preventing establishment of invasive species (Simpson et al. 2009, White et al. 2010). Because resources available for early detection networks are generally stretched thin, the identification of high priority targets, including high risk locations and routes for invasive species introduction, is critical to effective biosecurity (Paini and Yemshanov 2012, Colunga-Garcia et al. 2013).

The Gulf Coast of the United States has been identified as a region at risk for the emergence and establishment of ZIKV and other arboviruses associated with *Aedes* spp. mosquito vectors due to its warm and suitable climate as well as the presence of many international hubs of transportation (airports and seaports) within the region (Monaghan et al. 2016, Hotez and Jackson Lee 2017). The climate of this region is well within the climatic optima for transmission of ZIKV and other arboviruses (Tesla et al. 2018, Ryan et al. 2019), and is suitable habitat for both *Ae. aegypti* and *Ae. albopictus*, which are both commonly detected (Kraemer et al. 2015, Lounibos et al. 2016, Monaghan et al. 2016, McKenzie et al. 2019). However, while there have been outbreaks of ZIKV, DENV, and CHIKV in Gulf states (Graham et al. 2011, Kendrick et al. 2014, Likos 2016), these outbreaks do not compare in size or scope

with those experienced in nearby Latin America (Musso et al. 2018). This discrepancy may be at least partially explained by varying efforts towards vector control and public health between affected countries and the US, but many believe that they are also partially due to differences in vector competence between mosquito populations (Chouin-Carneiro et al. 2016, Azar et al. 2017, Ciota et al. 2017). Variation in vector competence between populations can occur at relatively fine scales (Alto et al. 2017, Azar et al. 2017), and may change with changes in gene flow caused by continuous reintroduction of vectors into a region. Thus, halting the genetic flow between disparate mosquito populations may aid in preventing the establishment of ZIKV, DENV, and CHIKV in the Gulf States region of the US. Developing a model that predicts reintroductions by *Ae. aegypti* and *Ae. albopictus* could help to alert public health officials of potential reintroductions and allow them to concentrate biosecurity efforts to limit dispersal and expansion (Paini and Yemshanov 2012). Furthermore, information on the origins of mosquitoes dispersing along these routes, combined with an understanding of genotypes and selective pressures among populations at either end, may allow researchers to predict changes in vector competence and insecticide resistance among populations experiencing immigration (Paini and Yemshanov 2012).

Because of the potential effects of long-distance dispersal by *Ae. aegypti* and *Ae. albopictus* on the transmission dynamics of associated arboviruses, it is crucial to understand dispersal patterns exhibited by these species. The goal of this study was to integrate available *Aedes* species distribution data and maritime movement data to identify ports along the Gulf Coast at high risk for importation of *Ae. albopictus* and *Ae. aegypti* via the GSN. Models representing maritime trade networks are common (Kaluza et al. 2010, Ducruet and Notteboom 2012, Colunga-Garcia et al. 2013), and have been implemented to determine likely origins of

insect invasions and to infer hotspots of invasion (Paini and Yemshanov 2012). However, this is the first model that integrates mosquito vector distribution data with maritime network data in order to provide a picture of vector dispersal along the GSN. This work may help officials to concentrate biosecurity efforts to prevent further mosquito invasion and potential importation of vector-borne pathogens in the Gulf Coast region of the US.

Methods

Data

We obtained data detailing every fully cellular container ship that arrived into major US ports on the Gulf of Mexico between January 1st and December 31st, 2012 from Informa (formerly Lloyd's Maritime Intelligence Unit; Informa, London, UK). These data were recorded by automatic identification system (AIS) transponders, which are installed on every large ship and at every port and canal in world. These transponders automatically report data on ship size, location, date, and other information each time a ship enters within range (~30 km) of another transponder. The dataset obtained from LMIU contained the previous ten ports of call for each ship before arriving in one of seven US ports and documents 1,921 arrivals and 204 departures of container ships, with many of these same ships arriving into the US Gulf ports multiple times within this period. These ships travelled to and from 213 ports in 69 countries. To ascertain the potential for invasion by *Ae. aegypti* and *Ae. albopictus* into each Gulf port, we used the distributions of these species provided by Kraemer et al. (2015) to identify which foreign ports within our network also hosted populations of these species.

Model

We used pathway-based, first-order Markov models to determine which ports along the US Gulf Coast were at the highest risk for importation of *Ae. aegypti* and *Ae. albopictus* along with container cargo shipped via maritime trade routes during the year examined, 2012. Given that a ship loads and unloads cargo with each stop, our models also assume that some potential exists for infestation of the ship by mosquitoes at each stop at a port occupied by these species. These models therefore assume that some transmission potential exists between each port occupied by these species, and all ports visited subsequently. Thus, given a route A-B-C-D, where point D is the final port of call in the Gulf of Mexico and B is a port where at least one species of mosquito is present, we assume some potential for transmission from B to C and then from C to D. Because there is also some probability of cargo containing the mosquitoes to be unloaded at each port, we considered all points on a route together, running from *i* to *j*. This information was then used to assemble a database of routes *i* to *j* and the number of trips made by vessels along these routes (Figure 1).

Each route, *i* to *j*, had an associated number of stops *ij*. Each port occupied by either *Ae. aegypti* or *Ae. albopictus* was assigned constant transmission potential, λ , which was used to calculate the potential for importation, P_{ij} , of each *Ae. aegypti* and *Ae. albopictus* into each one of our seven target ports:

$$P_{ij} = \lambda ij_1 + \lambda ij_2 + \dots \lambda ij_n$$

We then estimated the total relative likelihood of arrival by each species into each target port, ϕ_j , by summing P_{ij} for all trips into each target port:

$$\varphi_j = \sum P_{ij}$$

Sensitivity Analysis

Finally, we tested the sensitivity of our model parameterization by generating multiple values in 10% increments for our constant transmission potential, λ , and then generating a correlation matrix for φ_{ij} using Spearman rank correlation coefficients to ensure the resultant values were highly correlated. Both models were robust to changes in parameterization ($r_s > 0.964$).

Results

Within-Network Mosquito Distributions

Out of the 213 ports within our network, representing 69 countries, only 39 ports hosted neither populations of *Ae. aegypti* nor populations of *Ae. albopictus*. A total of 140 ports within our network were in locations with confirmed detection of *Ae. aegypti*, while 148 had confirmed detection of *Ae. albopictus*. A total of 114 ports within our network had known detection of both *Ae. aegypti* and *Ae. albopictus* (Figure 3).

Port Rankings

We determined that out of the seven target ports on the US Gulf Coast, Houston, TX was by far the most likely to receive cargo carrying *Ae. aegypti* or *Ae. albopictus*. New Orleans, LA and Mobile, AL were respectively identified as the ports with the next highest rankings, followed by Gulfport, MS, Freeport, TX, Tampa, FL and Galveston, TX (Table 1). We also found a strong correlation between the total number of cargo ship arrivals at each port and likelihood of arrival by both *Ae. aegypti* ($r^2 = 0.9986$, $P > 0.0001$) and *Ae. albopictus* ($r^2 = 0.9995$, $P > 0.0001$).

Of the 1,921 arrivals into target ports from January 1st to December 31st, 2012, a total of 1,905 had most recently visited ports occupied by both *Ae. aegypti* and *Ae. albopictus*. A further 6 arrivals had most recently visited ports occupied by only *Ae. aegypti* and 9 had most recently visited occupied by only *Ae. albopictus*. Only one arrival, which entered the port of Houston, had most recently visited a port where neither species is commonly found. The ports identified with the highest immediate connectivity to target ports in the Gulf were primarily ports on the Atlantic seaboard of the United States, in the Gulf of Mexico, or in the Caribbean (Table 2). We also observed a high level of connectivity between several target ports, especially Houston, New Orleans and Mobile (Table 3).

Discussion

The results of our model indicate a high degree of connectivity between US ports in the Gulf of Mexico. Our results also highlight the importance of a few ports, namely Houston and Tampa, which serve as the first ports-of-call for ships entering the US Gulf Coast network. High connectivity suggests great potential for dispersal of invasive *Aedes* spp. mosquitoes between ports on the US Gulf Coast, while the special connectivity of Houston and Tampa to outside ports suggests these may be important sites for biosecurity screening to prevent importation of invasive species such as these mosquitoes.

Network Characteristics and Potential Applications

The Port of Houston, TX represents by far the greatest risk for the dispersal of *Ae. aegypti* and *Ae. albopictus* to other US ports along the Gulf Coast. The Port of Houston received more than double the arrivals of fully cellular container ships in 2012 as did the port with the

next most arrivals, New Orleans. In fact, Houston received more arrivals during this period than did all six other major ports in the Gulf combined (Table 1). While more than three-quarters of container ships arriving in Houston had most recently come from a port outside the Gulf which host both *Ae. aegypti* and *Ae. albopictus*, the majority of traffic into other Gulf ports was internal, with arrivals coming from other Gulf ports (Table 2 and Table 3). These data are in line with other historical data on frequency of container ship arrivals and cargo tonnage, which show that Houston received more arrivals and handled more tonnage than any other port in the Gulf from 2016-2018, and that Houston handled a far higher proportion of foreign arrivals and freight than did other Gulf ports (US Department of Transportation: Bureau of Transportation Statistics 2018).

While the total number of arrivals by container ships may not always indicate the highest likelihood of arrival by invasive species, the broad distributions and common occurrence of *Ae. aegypti* and *Ae. albopictus* within our network led to a high correlation between these variables. Thus, we can assume that ports with the highest connectivity to target ports, the seven US ports along the Gulf of Mexico that were the target of our study, play a disproportionate role in the dispersal of invasive mosquitoes to our target ports. Because the probability of unloading infested cargo from a given port diminishes with each unloading visit along a cargo ship's route, and because accompanying invasive mosquitoes are most likely to survive and disperse given shorter travel times (Brown et al. 2017), we can assume that the ports most immediately visited by ships prior to arrival in US Gulf Ports pose the greatest relative risk for importation of *Ae. aegypti* and *Ae. albopictus*. This increased relative risk given lower distance (i.e. intermediate port visits) was included in our model, as first order Markov models are inherently weighted by distance (Abbeel and Ng 2005).

As the highest connectivity in our network was exhibited by several ports commonly visited immediately before arrival into our target ports, they are likely the greatest outside sources of invasive *Ae. aegypti* and *Ae. albopictus* dispersal into the US Gulf Coast. All of the five ports most highly connected to ports on the US Gulf Coast display especially high levels of connectivity with one particular port in the region. For instance, Altamira, Mexico, San Tomás de Castilla, Guatemala and Savannah, Georgia were highly connected to Houston, Texas while Puerto Cortes, Honduras was almost exclusively connected to Gulfport, Mississippi. While Kingston displayed the greatest evenness in terms of its connectivity to ports on the US Gulf Coast, nearly half of all trips made from Kingston to this region were to a single port, Tampa (Table 2).

While most ports on the US Gulf Coast have relatively little immediate connection to ports outside the Gulf Coast region (Table 2), the high level of connectivity between several US Gulf ports (Table 3) may provide a vehicle for dispersal of invasive species into ports with less outside connectivity. Because *Ae. aegypti* and *Ae. albopictus* are so widely distributed among port cities, and especially those connected to ports in the US Gulf Coast, implementation of origin-specific screening is unlikely to lead to increased efficiency in halting the dispersal of these species into the US Gulf Coast region. Instead, since US Gulf ports other than Houston have much lower levels of connectivity to ports outside the US Gulf Coast and since there is a high level of connectivity between Gulf ports, preventing mosquitoes from outside the US Gulf Coast from entering the US Gulf Coast network seems both particularly critical and relatively simple. Since Houston serves as a hub for vessels entering the US Gulf Coast network, implementation of an early alert and rapid response system for screening ships entering the Port

of Houston could disproportionately reduce the risk of maritime dispersal of invasive species, including *Ae. aegypti* and *Ae. albopictus*.

These models may provide a basis for further research regarding movement of mosquito vectors and their associated pathogens via the GSN. Collection of vector species at key ports within this network and examination of the population genetics at these locations could provide validation for this model. An examination of these populations' genetics may also help to identify the relative influence of maritime trade and other processes, such as overland dispersal and selective pressures, in determining local population genetics.

In the past several decades we have seen an exponential upsurge in *Aedes* spp. associated arboviruses around the world, partially due to the success and expansion of *Aedes* spp. vector mosquitoes (Kraemer et al. 2019, Brady and Hay 2020). The range and incidence of DENV have been increasing for decades, with an estimated 400 million cases a year and over one-sixth of the world's population at risk each year (Bhatt et al. 2013). Yellow fever virus has also reemerged as a major infection, causing sizeable epidemics in South America and Africa. (Grobbelaar et al. 2016, Barrett 2018, Giovanetti et al. 2019). Along with the reemergence of YFV and DENV, we have seen the introduction of CHIKV and ZIKV to the Western Hemisphere, and associated outbreaks and epidemics across this new range (Fauci and Morens 2016, Yactayo et al. 2016). The emergence and reemergence of these arboviral threats is tied to the establishment and success of competent populations of *Aedes* spp. mosquitoes (Kraemer et al. 2019), so understanding the movements of these species, as well as potential gene flow caused by dispersal, may help to turn the tide against the global rise of the arboviruses.

Technical Aspects of the Pathway Models

Because no future forecast was imposed upon this AIS data, our models are best interpreted as showing contemporary potential for movement of *Ae. aegypti* and *Ae. albopictus*, rather than as a projection of the future. While our results accurately reflect the movements of all fully cellular container ships that arrived in the 7 target ports along the US Gulf Coast, a number of potential routes of dispersal and potential vectors for dispersal were not considered in our study. Our data did not include any information on the movements of non-containerized cargo along the GSN. The actual roles played by these different cargo ship types in the dispersal of container-breeding mosquitoes is unknown. However, container ships are often considered to be better vectors for the dispersal of terrestrial invasive species because containers are often not opened and examined between destinations (Derraik 2004, Paini and Yemshanov 2012). Container ships also tend to follow relatively predictable routes, which are repeated year after year, while bulk dry goods ships and other cargo carriers move less predictably and according to demand, making their movements more difficult to model or predict (Kaluza et al. 2010).

The potential role of airports and air travel in mosquito dispersal was also not included in our model. While air travel is thought to play a major role in the spread of invasive species in general (Early et al. 2016), and disease vector species in particular (Tatem, Hay, et al. 2006, Tatem et al. 2012), maritime travel is thought to play a much larger role in the dispersal of *Aedes* spp. mosquitoes due to unique aspects of their ecology (Benedict et al. 2007, Kraemer et al. 2019, Brady and Hay 2020). The eggs of both *Ae. aegypti* and *Ae. albopictus* can enter diapause during periods of environmental stress, including extreme temperatures and desiccation. These eggs may then survive for months under these conditions, eclosing only when the environment

becomes more favorable (Hawley 1988, Estrada-Franco and Craig 1995, Brown et al. 2017). This ability to undergo diapause makes these species ideally suited to maritime travel.

Our models and results also did not account for the potential role played by dispersed ports and anchorages, which are collections of multiple anchorages capable of hosting, at most, a few container ships at a time. Data on these dispersed anchorages tends to be reported separately, even if anchorages are within a few miles of each other, because each anchorage hosts its own AIS recorder (Kaluza et al. 2010). While individual anchorages may not host a great capacity of ships, or individually contribute much to the passage of goods and invasive species along the GSN, dense conglomerations of anchorages, such as that found in South Louisiana at the headwaters of the Mississippi River, actually handle large numbers of ships and cargo tonnage each year (Kaluza et al. 2010, Ducruet and Notteboom 2012). The conglomeration of South Louisiana anchorages, for instance, handles the highest tonnage of cargo each year of any American port. However, the majority of cargo handled by dispersed anchorages, including South Louisiana, tends to be dry bulk goods, such as grains and coal (Kaluza et al. 2010, US Department of Transportation: Bureau of Transportation Statistics 2018), and the ships that carry these goods were not included in the study. Container ships, which were the type of ship examined in our models, rarely load or unload cargo at these anchorages, so stops there are typically just delays which do not pose much opportunity for dispersal of invasive terrestrial species (Stergiopoulos et al. 2018).

Our model also contains several assumptions and generalizations necessitated by data availability and the general lack of knowledge regarding transport of mosquitoes in cargo. Data collected by AIS does not include information on the number of containers or the type of cargo carried by each ship, so we assumed that each ship had the same capacity for infestation and

transmission. These records also do not include information on whether cargo was loaded or unloaded at each port. Some port visits are made for purposes of refueling and involve no transfer of cargo to or from the vessel (Ducruet and Notteboom 2012). Additionally, there is little information on how often mosquitoes such as *Ae. aegypti* or *Ae. albopictus* may infest cargo vessels. While many sources detail dispersal of these species by importation of used tires, plants, and other cargo (Reiter and Sprenger 1987b, Hofhuis et al. 2009, Pliego Pliego et al. 2018), these sources do not provide quantification of dispersal or infestation. Because of these, we assumed a constant probability for transmission from one port where a mosquito occurred to the next port, while in reality this probability is certainly heterogenous. More detailed information containers and cargo, as well as quantification of mosquito infestation of these cargo, would dramatically improve this model and provide more insight into paths utilized by *Ae. aegypti* and *Ae. albopictus* for dispersal.

Conclusion

This study represents the first pathway-based analysis of dispersal by *Ae. aegypti* and *Ae. albopictus* into and among major ports on the US Gulf Coast via the GSN. These mosquitoes, which are the primary vectors of numerous arboviruses (Paupy et al. 2009, McKenzie et al. 2019, Souza-Neto et al. 2019), are also some of the most invasive insects on earth (Benedict et al. 2007, Bonizzoni et al. 2013, Kraemer et al. 2019). Establishment of these species is critical for transmission of CHIKV, DENV, YFV, and ZIKV, all emerging and reemerging arboviral threats that have shaken the world in recent decades. Understanding long-distance dispersal of these species via maritime trade allows us to concentrate biosecurity and vector control efforts, and may allow us to better understand gene flow and patterns of population genetics as well as

associated phenotypic traits that are important for mosquito control and public health (Manni et al. 2017). Future studies should consider population structure and potential metapopulation dynamics between invasive mosquito populations in highly connected port cities in order to understand how these are affected by maritime dispersal. This study may serve as a model for future studies of dispersal invasive species, including other mosquito species, via maritime trade. A number of highly invasive and medically important mosquitoes, including *Anopheles stephensi* (Liston), *Aedes (Hulecoeteomyia) koreicus* and *Aedes (Finlaya) japonicus japonicus* (Theobald), are currently expanding their global ranges both over land and through long-distance dispersal via the GSN (Medlock et al. 2012, Kaufman and Fonseca 2014, Surendran et al. 2019). By understanding vector dispersal and its downstream effects, we may better understand and prevent outbreaks of vector-borne pathogens across the world.

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Figures and Tables

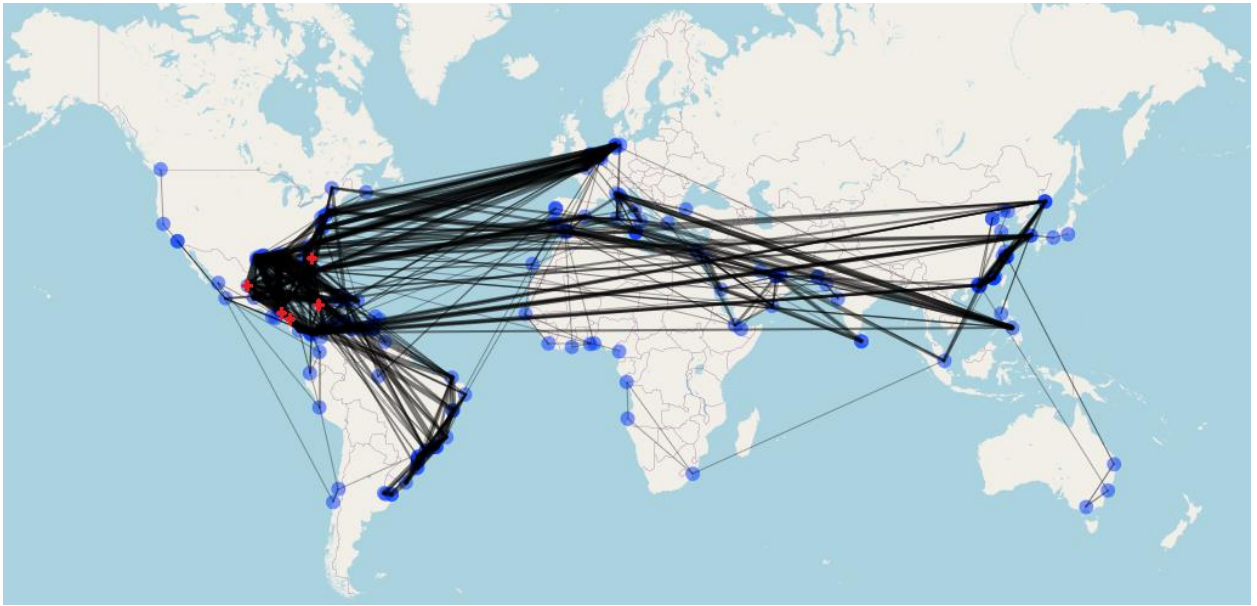


Figure 1. Our network consisted of 213 ports, representing 69 countries. Lines are logarithmically weighted to demonstrate connectivity between ports. The five outside ports with the greatest connectivity to ports in the Gulf are marked in red.

Table 1. Ports along the Gulf Coast of the US with the highest relative likelihood of arrival (ϕ_j) by *Aedes aegypti* and *Ae. albopictus* via the international maritime trade network given a constant transmission potential (λ) of 0.5. The total number of arrivals of fully cellular container ships at each port, from January 1st to December 31st, 2012, was strongly correlated with relative likelihood of arrival by both *Ae. aegypti* ($r^2 = 0.9986$, $P > 0.0001$) and *Ae. albopictus* ($r^2 = 0.9995$, $P > 0.0001$) during this time frame.

| | ϕ_j (<i>Aedes aegypti</i>) | ϕ_j (<i>Aedes albopictus</i>) | <i>Total maritime trade traffic (container ship arrivals)</i> |
|------------------------|-----------------------------------|--------------------------------------|---|
| <i>Houston, TX</i> | 885.75 | 909.39 | 985 |
| <i>New Orleans, LA</i> | 422.34 | 420.30 | 435 |
| <i>Mobile, AL</i> | 236.73 | 240.07 | 252 |
| <i>Gulfport, MS</i> | 132.52 | 135.54 | 136 |
| <i>Freeport, TX</i> | 54.95 | 54.52 | 55 |
| <i>Tampa, FL</i> | 55.93 | 50.51 | 50 |
| <i>Galveston, TX</i> | 1.99 | 1.85 | 2 |

Table 2. Outside ports with the highest immediate connectivity to our seven target ports in the US Gulf States. Since nearly all maritime arrivals in the Gulf passed most recently through ports on the Atlantic seaboard, in the Caribbean or in other ports on the Gulf of Mexico, all of which host populations of both *Aedes aegypti* and *Aedes albopictus*, mosquito populations from these ports must reasonably be assumed to be the most likely to arrive in target ports. Each trip represents one directed network segment between ports. Data represents arrivals by fully cellular container ships from January 1st to December 31st, 2012.

| | Total trips to target ports | Trips to Houston | Trips to New Orleans | Trips to Mobile | Trips to Gulfport | Trips to Tampa |
|---|------------------------------------|-------------------------|-----------------------------|------------------------|--------------------------|-----------------------|
| Altamira, Mexico | 373 | 364 | 0 | 9 | 0 | 0 |
| Santo Tomás de Castilla, Guatemala | 160 | 130 | 29 | 0 | 1 | 0 |
| Puerto Cortes, Honduras | 105 | 3 | 5 | 0 | 96 | 0 |
| Savannah, Georgia, USA | 104 | 102 | 0 | 2 | 0 | 0 |
| Kingston, Jamaica | 102 | 17 | 18 | 18 | 0 | 49 |

Table 3. High connectivity between ports on the US Gulf Coast implies high risk for movement of *Aedes* spp. mosquitoes between these cities. While Houston seems to play a role as a hub for international arrivals, New Orleans and Mobile receive a great number of shipments from domestic ports, including Houston. Each trip represents one directed network segment between ports. Data represents arrivals by fully cellular container ships from January 1st to December 31st, 2012.

| | Trips to Houston | Trips to New Orleans | Trips to Mobile | Trips to Gulfport | Trips to Freeport | Trips to Tampa | Trips to Galveston |
|------------------------|-------------------------|-----------------------------|------------------------|--------------------------|--------------------------|-----------------------|---------------------------|
| Houston, TX | NA | 316 | 98 | 0 | 6 | 0 | 2 |
| New Orleans, LA | 89 | NA | 50 | 0 | 0 | 0 | 0 |
| Mobile, AL | 32 | 39 | NA | 0 | 0 | 7 | 0 |
| Gulfport, MS | 0 | 0 | 0 | NA | 0 | 0 | 0 |
| Freeport, TX | 0 | 6 | 0 | 0 | NA | 0 | 0 |
| Tampa, FL | 0 | 0 | 48 | 0 | 0 | NA | 0 |
| Galveston, TX | 2 | 0 | 0 | 0 | 0 | 0 | NA |