ECOLOGICAL AND EVOLUTIONARY DRIVERS OF INVASION SUCCESS IN A MOSQUITO

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Ecology and Evolution

written under the direction of

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New Brunswick, New Jersey

May 2014
ABSTRACT OF THE DISSERTATION

Ecological and evolutionary drivers of invasion success in a mosquito

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The label ‘invasive’ is most often applied to exotic species that have established and spread, frequently becoming so abundant that they have negative impacts on humans and ecosystems. Understanding what factors promote invasiveness may allow us to better manage the impacts of exotic species. In my dissertation I strive to elucidate ecological and evolutionary processes responsible for the success of an emerging invasive mosquito, Aedes japonicus japonicus, a cold-adapted species from Asia with established populations in Europe, North America, and surprisingly, in sub-tropical Hawaii.

In Chapter 1, I report the results of laboratory experiments examining the interactions between larvae of Ae. j. japonicus and another invasive mosquito, Culex quinquefasciatus, and associated microbial fauna. In a high temperature experiment, Ae. j. japonicus only survived when Cx. quinquefasciatus, a tropical and pollution-tolerant species, was present. Treatments with Cx. quinquefasciatus contained significantly lower numbers of a protozoan flagellate that is potentially toxic to Ae. j. japonicus. From these findings I speculate that some invasive mosquitoes can ameliorate habitat conditions allowing other species to exploit new geographic areas and microhabitats, the first time that facilitation between mosquitoes has been proposed.
In Chapters 2 and 3, I describe the genetic structure of *Ae. j. japonicus* across elevational gradients in Hawaii and Virginia, respectively. In Hawaii, populations at warmer low elevations display signatures of bottlenecks, including lower genetic diversity and greater genetic differentiation, which support the findings from Chapter 1 that this species survives poorly at warm temperatures. In Virginia, I also observed elevational differences in genetic patterns consistent with temperature-mediated selection, though frequent long-distance dispersal events (probably human-mediated transportation along roads) augment genetic diversity within low elevation populations.

In summary, I postulate that *Ae. j. japonicus* has profited from habitat amelioration by co-occurring mosquitoes and from long-distance transport by humans. In fact, I hypothesize that humans have driven the post-establishment evolution of invasiveness in this mosquito, both by increasing genetic diversity through population admixture and by exposing it to novel selective pressures. These results underline the importance of preventing multiple introductions and restricting gene flow between exotic populations in order to limit their evolutionary potential.
Acknowledgements

I would like to thank my advisor, Dr. Dina Fonseca, for her immense contributions to my intellectual and personal development during my time at Rutgers; my committee, Dr. Peter Smouse, Dr. Peter Morin, Dr. Shannon LaDeau, and Dr. Daniel Strickman, for always being willing to answer my questions and provide feedback on my projects; Dr. Nina Fefferman, for valuable advice and stimulating conversations; Marsha Morin, for always knowing the answer to everything; Kristina Carle and Linda McCuiston, for keeping the Center for Vector Biology and its six-legged inhabitants up and running; other graduate students in both the E&E and Entomology graduate programs for providing much needed laughs, encouragement, and commiseration; my family, for always believing in me and maintaining an interest in my work; Dana, for being wonderful and keeping me sane; and Bowser, for unlimited puppy kisses and snuggles.

I would also like to acknowledge my funding sources: TA support from Rutgers SEBS and DLS-SAS; GA support from Rutgers start-up funds to Dr. Fonseca and Cooperative Agreement USDA-ARS-58-6615-8-105; a Pre-Dissertation Award from the Rutgers-New Brunswick Graduate School; the Buell Award from the Rutgers Dept. of Ecology, Evolution and Natural Resources; an E&E Small Grant from the Rutgers Ecology & Evolution Graduate Program; and the Jobbins Scholarship from the Northeastern Mosquito Control Association.
Acknowledgement of publication

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Introduction

The term ‘invasive,’ while a subject of ongoing debate (Richardson et al. 2000, Colautti and Maclsaac 2004, Valéry et al. 2008), is most often applied to an aggressively expanding exotic species that is present in sufficiently large numbers to pose a threat to native ecosystems and human enterprise (as in Lockwood et al. 2007). Several factors have been proposed as determinants of invasion success: rapid growth, large reproductive capacity, ease of dispersal, superior competitive ability, and the size and frequency of introductions (propagule pressure) (Ricciardi and Rasmussen 1998, Sakai et al. 2001, Lockwood et al. 2005). While not all exotic species become invasive, many that do are probably overlooked (due to their small size, e.g. microbes) and their impact may be much larger than we realize. By contrast, the arrival of a new mosquito (Diptera: Culicidae) is often detected early, largely because most developed countries have established vector surveillance programs, in response to the disease threat posed to humans and economically important livestock. Invasive mosquitoes may also draw attention to themselves by nuisance biting (Benedict et al. 2007). Because their establishment and spread is often well documented, mosquitoes make excellent models for answering questions about how species become invasive.

Dozens of mosquito species have successfully established in areas outside their native ranges, though most remain limited in distribution (as small as a single island in some cases) and are not known to have strong negative effects on either native organisms or humans (Juliano and Lounibos 2005). A handful of species, however, have dramatically increased in range and abundance to become notorious worldwide invaders. The latter group of species includes *Aedes (Stegomyia) aegypti* (Linnaeus), native to
Africa and currently found across the world’s tropics; *Culex (Culex) pipiens* (Linnaeus), native to the Old World and currently found in urban North America, South America, and Australia; *Culex (Culex) quinquefasciatus* (Say), native to the Old World’s tropics but currently a pan-tropical invader; and *Aedes (Stegomyia) albopictus* (Skuse), native to temperate and tropical Asia and currently found in the Americas, Europe and Africa (Christophers 1960, Hawley 1988, Vinogradova 2000a). These particular species have caused major problems by virtue of their ability to vector human and wildlife disease (Juliano and Lounibos 2005). Introduced *Ae. aegypti* vectored the Yellow Fever and Dengue outbreaks that plagued the Americas until the mid-twentieth century, when the advent of DDT drastically reduced worldwide mosquito populations (Gubler 2004). The recent spread of *Ae. albopictus* accompanying worldwide reductions in insecticide use has contributed to a reemergence of many of these same diseases and has spread new ones, e.g., the recent outbreaks of Chikungunya in Italy (Rezza et al. 2007), as well as Dengue in Hawaii (Effler et al. 2005) and the Florida keys (2010). The spread and growth of *Ae. albopictus* populations exerted strong selective pressure on the Chikungunya virus, formerly vectored by *Ae. aegypti*, which then developed mutations in the viral envelope increasing the vector competence of *Ae. albopictus* and resulting in new epidemics (Ng and Hapuarachchi 2010, Tsetsarkin and Weaver 2011). Introduced mosquitoes also present a major threat to native wildlife: in Hawaii, avian malaria spread by *Cx. quinquefasciatus* has been strongly implicated in the extinction of many of the endemic Hawaiian honeycreepers (Warner 1968, van Riper et al. 1986).

What determines whether an exotic mosquito will stay localized and inert, or expand aggressively and become invasive? One factor that has been proposed to
contribute to invasion success is the ability to outcompete native species and other established exotics in the new range. This has been extensively examined in the case of *Ae. albopictus* in the U.S. (Juliano and Lounibos 2005), the most recent and well studied mosquito invasion. Interspecific competitive theory predicts that the species with the lowest R* (resource density at which it is able to maintain positive population growth) will competitively exclude the other species (Monod 1950). Both lab (Barrera 1996) and field (Juliano 1998, Juliano et al. 2004) experiments confirm that *Ae. albopictus* is able to maintain positive population growth at a lower food availability than *Ae. aegypti*, resulting in competitive exclusion of *Ae. aegypti*. Fittingly, the spread of *Ae. albopictus* in Florida was associated with declines in *Ae. aegypti* populations (O'Meara et al. 1995). *Ae. albopictus* has also been shown to outcompete other resident species, depending on experimental conditions like the type and amount of food resources, habitat type, and presence of insecticides or predators (Novak et al. 1993, Costanzo et al. 2005, Kesavaraju et al. 2008, Costanzo et al. 2011, Kesavaraju et al. 2011, Allgood and Yee 2014, Kesavaraju et al. 2014).

A second trait significantly more common among invasive mosquitoes than in non-invasive exotics is the ability to exploit human-dominated ecosystems (Juliano and Lounibos 2005). Their larvae can develop in a wide variety of small containers in urban areas (e.g., discarded tires, potted plant trays, garbage cans, pieces of trash, anything that can collect water). Many are highly tolerant of pollution, and some species, particularly *Cx. quinquefasciatus*, can develop in raw sewage (Singh 1967, Vinogradova 2000a). A subspecies of *Cx. pipiens*, *Cx. pipiens pipiens f. molestus*, has adapted to humans so well that it lives almost exclusively underneath cities (Byrne and Nichols 1999) and has
diverged genetically from above-ground populations (Fonseca et al. 2004). Adults of all these species utilize humans as blood sources, and in some cases, feed mainly (ex. *Ae. albopictus*, Egizi et al. 2013) or even exclusively (ex. *Ae. aegypti*, Ponlawat and Harrington 2005) on humans. Such combinations of traits yield highly adaptive human commensals, and have almost surely contributed to their wide ranging distributions, for example by making them more likely to be transported by humans. Historically, invaders such as *Cx. pipiens*, *Cx. quinquefasciatus*, and *Ae. aegypti* were commonly spread as larvae in ship ballast and drinking water. The more recent expansion of *Ae. albopictus* (reaching the mainland US in the mid 1980’s, Sprenger and Wuithiranyagool 1986) has been linked to the global-scale used tire and potted plant trade. Its eggs can withstand temporary dry spells during shipments, hatching in the new location following exposure to water (Reiter 1998), an adaptation ideal for human transport.

*Aedes (Finlaya) japonicus japonicus* (Theobald), the Asian bush mosquito (Cameron et al. 2010), is the latest mosquito invader to make its way across the globe. One of the 4 subspecies of *Aedes japonicus* (sometimes called *Ochlerotatus japonicus*, following Reinert 2000), it is a cold-adapted species native to northern Japan and Korea (Tanaka et al. 1979). In recent years it has expanded primarily across northern latitudes in the US (Peyton et al. 1999, Roppo et al. 2004), Canada (Thielman and Hunter 2006), and northern Europe (Schaffner et al. 2009). It has also begun to spread into the southern U.S. along the Appalachian mountains (Reeves and Korecki 2004). In eastern North America, its range has increased from 4 U.S. states to 29 states and 2 Canadian provinces in only 15 years (Kaufman and Fonseca 2014). Among mosquitoes, the extent and speed of its spread rivals that of *Ae. albopictus*, considered one of the world’s 100 worst
invaders (Benedict et al. 2007). Unlike *Ae. albopictus*, however, there is relatively little support for the idea that *Ae. j. japonicus* is a superior competitor (Kaufman and Fonseca 2014) and it appears to have less of an association with humans in its native range, exploiting more forested habitat in Japan and rarely feeding on humans (Tanaka et al. 1979). Instead, it has been hypothesized that *Ae. j. japonicus*’ tolerance to cold (Scott 2003) may allow it to exploit an underutilized niche, with an extended window of activity (both earlier and later in the season), as well as the use of comparatively cooler microhabitats (Bartlett-Healy et al. 2012) than other container-occupying species (Kaufman and Fonseca 2014). Interestingly, despite being cold-adapted and largely restricted to cooler latitudes elsewhere in the world, *Ae. j. japonicus* has been able to establish a population on the island of Hawaii (Larish and Savage 2005). Hawaii’s climate is stratified by elevation: tropical at low elevations but with cold refugia higher up on the volcanoes, and with little seasonal variation in temperature. *Aedes j. japonicus* was first collected in Laupahoehoe, a low elevation town on the northern coast of the island of Hawaii in 2004 (Larish and Savage 2005). It has now spread across much of the island, particularly at high elevation, although it remains limited to the island of Hawaii (Larish et al. 2010). *Aedes j. japonicus* frequently shares larval habitat in Hawaii with *Cx. quinquefasciatus*, another invasive mosquito, and a tropical species (Egizi AE, personal observation). Depending on their outcome, species interactions can either impede or promote invasion success (Lockwood et al. 2007), and it is possible that *Ae. j. japonicus* has been influenced in its spread by co-occurring species. Additionally, it may have undergone genetic change in response to new environments such as the warmer temperatures at low elevation, as there is accumulating evidence that evolution can occur
quickly in invasive species (Lee 2002).

The establishment of *Ae. j. japonicus* on Hawaii presents a good model system for studying the ecology and evolution of biological invasions. To understand the role of species interactions in invasion success I will examine the interaction of *Ae. j. japonicus* with *Cx. quinquefasciatus*, and with container-inhabiting microbes (Chapter 1). I will also examine spatial patterns of genetic diversity and differentiation across populations of *Ae. j. japonicus* in Hawaii and in the mainland US (Virginia), attempting to elucidate critical processes in the evolution of newly introduced populations (Chapters 2 and 3).
Chapter 1:

Unraveling microbe-mediated interactions between mosquito larvae in a laboratory microcosm

Abstract

Interspecies interactions have important impacts on communities and when multiple trophic levels are involved effects can be complex and indirect. For mosquitoes, interactions experienced as larvae affect adult attributes such as survivorship, reproductive output, and longevity, factors that can affect their ability to vector disease. We examined how larvae of two ecologically distinct mosquito species, *Aedes japonicus japonicus* and *Culex quinquefasciatus*, interact at different temperatures (17°C and 27°C) and at different relative densities. We also quantified abundances of bacteria and protozoan flagellates to uncover how changes in the microbial community affect the outcome of the two mosquitoes’ interaction. At 17°C, survival and size of both mosquito species was not affected by the other’s presence. *Cx. quinquefasciatus* was strongly affected by intraspecific, but not interspecific, competition at both temperatures. At 27°C, *Ae. j. japonicus* larvae experienced 100% mortality in treatments by themselves and treatments where *Cx. quinquefasciatus* was abundant, surviving only in the presence of low densities of *Cx. quinquefasciatus*. Both the total bacteria count and counts of a protozoan flagellate identified as *Spumella* spp. decreased with increasing numbers of *Cx. quinquefasciatus*. We postulate that at 27°C, the survival of *Ae. j. japonicus* depends on the interaction between *Cx. quinquefasciatus* and the microbial community. This study demonstrates that one mosquito species may alter the microbial community in ways that indirectly influence another mosquito species’ larval survival, and by extension adult abundance and potential disease transmission.
Introduction

Ecological species interactions range from those resulting in negative outcomes, such as competition and predation, to those that have positive outcomes such as mutualism and commensalism. Interactions can be direct, meaning between two species only, or indirect, when two species affect each other through a third (or more) intermediary species (Wootton 1994). Their effect on the third species can either be to alter its abundance (interaction chain indirect effect) or to alter how it interacts with the other species (interaction modification indirect effect), for example by changing its behavior (Wootton 1994). Indirect effects are important to consider because they can have far-reaching impacts throughout food webs, affecting multiple species in the community.

Communities within small water-filled containers often include immature stages (larvae) of mosquito species. These containers, whether natural (e.g. treeholes) or manmade (e.g. tires and buckets), accumulate fallen leaves and detritus that serve as a resource base for microorganisms, which in turn are consumed by suspension feeders such as mosquito larvae. Importantly, larval resource availability affects characteristics of the adult mosquito such as size, abundance, and local occurrence, all of which are known to influence disease dynamics. For example, high levels of competition between mosquito larvae produce smaller adults with a decreased ability to transmit disease due to reduced longevity (Grimstad and Walker 1991, Alto et al. 2005, Alto et al. 2008).

Because of the relevance of mosquito borne diseases to public health, as well as the relative ease of mimicking small container communities in the laboratory, many studies have examined interspecies interactions among larval mosquitoes. These studies
typically find that the species compete for food as larvae, affecting population parameters such as adult size, survivorship, and time to emergence (Juliano 2009). The food base that they compete for is microbes – primarily bacteria, protozoa, fungi, and algae found on the sides of containers, on detritus, and in the water column (Merritt et al. 1992). Laboratory and field experiments have examined how mosquitoes modify the microbial community and found that in addition to reducing overall abundance of microorganisms, they can also change the community composition of bacteria (Kaufman et al. 2000, Evans 2007), protozoans (Addicott 1974, Gray et al. 2006), and algae (Gimnig et al. 2002). Further, different species of mosquitoes can have different effects on microbial communities. For example, a recent study found large numbers of Enterobacteriaceae in Culex tarsalis guts collected outdoors (Duguma et al. 2013), suggesting they remove this group of bacteria from the water column via feeding. By contrast, in a laboratory experiment the proportion of Enterobacteriaceae in the water actually increased when Aedes triseriatus were added (Kaufman et al. 1999). Therefore in addition to direct competition for the same food resources, co-occurring mosquitoes may also be able to affect each other indirectly through differential modification of the microbial community.

*Culex quinquefasciatus* (Say) and *Aedes japonicus japonicus* (Theobald) are two mosquito species with very different ecological niches. *Culex quinquefasciatus*, known as the southern house mosquito, occurs throughout the world’s tropical and subtropical regions (approximately between latitudes 36°N and 36°S) (Vinogradova 2000b, Fonseca et al. 2006) and as a tropical species its females lack the ability to enter winter diapause (Eldridge 1968). A laboratory experiment testing larval development at a range of temperatures from 15-34°C found the highest survival to adulthood at 25°C (Rueda et al.
1990). *Cx. quinquefasciatus* is also notorious for thriving in highly polluted water (Vinogradova 2000b), and it is not uncommon to find them breeding in raw sewage (Raghavan 1961, Singh 1967, Subra 1981). In fact, sewage overflow with high concentrations of ammonia and phosphorus can lead to increased development rates and larger body size relative to unpolluted habitats (Chaves et al. 2011, Lund et al. 2014). Field densities of *Cx. quinquefasciatus* can be high, particularly in polluted environments, ranging from 300 to 1000 larvae/L (Sunahara et al. 1998, Chaves et al. 2011). In this species parameters like body size and development time are more commonly affected by intraspecific than interspecific competition, characteristic of a superior competitor (Mohsen and Al-Saady 1995, Smith et al. 1995, Agnew et al. 2000).

By contrast, *Ae. j. japonicus*, also known as the Asian bush mosquito, is a cold-adapted species native to northern Japan and Korea (Tanaka et al. 1979). In recent years it has also expanded across northern latitudes in the US (Peyton et al. 1999, Roppo et al. 2004), Canada (Thielman and Hunter 2006), and northern Europe (Schaffner et al. 2009). To survive the winter *Ae. j. japonicus* females lay eggs in the fall that do not hatch until temperatures increase in the spring (Tanaka et al. 1979), although in relatively mild winters, larvae can survive for months at low temperatures (Scott 2003). In laboratory experiments with temperatures ranging from 10-40°C the highest larval survival occurred at 16°C (Scott 2003). While the pollution tolerance of *Ae. j. japonicus* has not been tested experimentally, observations of larval habitat in its native and invasive range indicate a species that favors cleaner habitats than *Cx. quinquefasciatus*. It is most often observed in containers with clear water and leaf detritus, such as rock pools and rain barrels (Feng 1938, Tanaka et al. 1979, Scott et al. 2001). A survey in its invasive range found
relatively low larval densities (0.2 to 41.5 larvae/L, Bartlett-Healy et al. 2012), although there is evidence it may occur at somewhat higher densities in its native range (50 to 450 larvae/L, Sota et al. 1994). Laboratory competition experiments have revealed that *Ae. j. japonicus* larvae are similarly affected by intra- and interspecific competition and it is not considered a strong competitor (see Kaufman and Fonseca 2014 for a review).

Despite their nearly opposite geographic distributions, the two species now co-occur on the island of Hawaii, where *Cx. quinquefasciatus* has been present since the 1820’s (Warner 1968) and *Ae. j. japonicus* recently arrived in 2003 (Larish and Savage 2005). The introduction of *Ae. j. japonicus* into Hawaii evokes a number of questions about how these ecologically distinct species might interact. Based on their known ecology we hypothesized that their interaction would be affected by temperature and each species’ relative abundance. Specifically, we expected the tropical species, *Cx. quinquefasciatus* to be the stronger competitor at higher temperatures and when in greater abundance. To test these hypotheses we examined their interaction at two different temperatures, using a replacement series design (Novak et al. 1993) to control for density dependent effects, and used an artificial laboratory microcosm in order to control key variables, like temperature, food levels, and light regime. Further, we attempted to unravel the mechanisms underlying their interaction by quantifying bacterial and protozoan densities and identifying the most abundant flagellates in the treatments.

**Materials and methods**

*Replacement series experiments*

We obtained *Aedes j. japonicus* and *Cx. quinquefasciatus* eggs from colonies maintained at the Center for Vector Biology at Rutgers University, which were started in
2008 and 2005, respectively. Hatching was synchronized by exposing *Ae. j. japonicus* eggs to water after *Cx. quinquefasciatus* egg rafts were laid, and eggs from both species were allowed to hatch for 24 h prior to the start of the experiment at room temperature. One hundred 1<sup>st</sup> instar larvae were added to experimental cups in the following ratios: 0 *Cx. quinquefasciatus* (Q): 100 *Ae. j. japonicus* (J), 25Q:75J, 50Q:50J, 75Q:25J, and 100Q:0J. We used 250ml plastic cups filled with 200ml tap water as experimental units to mimic the small containers these species commonly utilize in the field. The cups were then transferred to two incubators (IN024 Insect Growth Chambers, Darwin Chambers, St. Louis, MO) kept on a 12:12 L:D cycle, one set at 17°C and the other at 27°C. We chose these temperatures because they are similar both to (1) summer temperatures in Hawaii at mid (16-18°C, 1210m) and low (24-26°C, 11m) elevations (Ahumada et al. 2004) and (2) temperatures at which *Ae. j. japonicus* and *Cx. quinquefasciatus* experienced differentially high survivorship in laboratory experiments (Rueda et al. 1990, Scott 2003).

We performed three replicates of each treatment combination (5 species ratios X 2 temperature levels) for a total of 10 treatment combinations and 30 containers. To account for any bias of incubator location, cups were arranged such that one replicate of each treatment was on each of three shelves. Each cup was given 0.05g of Purina Rat Chow (Purina Mills LLC, Gray Summit, MO) on the 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, and 9<sup>th</sup> days to provide a resource for microbial growth. While rat chow is not a typical resource base in natural container communities it does provide the required nutrients for larval development and is commonly used to rear laboratory colonies of both species (L. McCuiston personal communication, Smith et al. 1995, Williges et al. 2008). We checked water levels daily.
and added new water to the containers as needed to compensate for evaporation and keep water levels constant. On the 7th day due to the high mortality and cloudy appearance of *Ae. j. japonicus* treatments at 27°C (see Results) we decided to postpone the next feeding until the 9th day. This change was performed across all treatments and in all subsequent experiments.

Cups were checked daily for pupae, which were removed and transferred to emergence containers designed to prevent the newly emerged adults from falling into the water and drowning. After emergence, adults were killed by exposure to -80°C for five minutes, then transferred to desiccation containers consisting of labeled petri dishes resting on top of desiccant (DampRid, Inc., a W.M. Barr company, Memphis, Tennessee) in a sealed plastic container. Adults were kept in these desiccation containers until the end of the experiment. All adults had emerged from the 27°C treatment after 19 days, while they continued to emerge from the 17°C treatment until the 49th day. After all mosquitoes from both treatments had emerged, the number of male and female adults from each cup was counted for each species. Adult female wing length is typically measured as a proxy of female fecundity in mosquitoes (Packer and Corbet 1989). To measure female wing lengths we removed one wing from each emerged female, affixed it to a microscope slide with clear tape, and photographed it using a Nikon SMZU and a Nikon DXM1200C microscope-attached camera (Nikon Instruments, Inc, Melville, NY). Then we measured the length from the alular notch to the furthest distal margin of the wing, excluding fringe scales, using the line measuring feature in ImageJ (W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA, [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij), 1997-2011).
After completion of the first experiment and discovery that the two species were affected by each other at 27°C, but not 17°C (see Results), we repeated the 27°C treatment by itself and included measurements of bacterial and protozoan abundances across treatments to examine how the microbial community might be indirectly mediating their interaction. The protocol from the first experiment was duplicated with the exception that the microbial community was standardized at the beginning of the experiment in the following manner: first, we created a microbial culture by adding 0.05 g of rat chow to 200ml tap water on the 1st, 3rd, 5th and 9th day (following the same protocol as the first experiment without the addition of mosquitoes), allowing microbes entering from the air, water, or rat chow to proliferate for a total of 11 days; second, the water added to the treatment cups was first filtered through a Corning 0.22µm filter; third, 2ml of the microbial culture was added to each treatment. Six replicates of each species ratio treatment were initiated, three of which were “sacrificed” five days into the experiment to allow for direct counts of protozoa and bacteria without altering conditions in the remaining cups.

Analysis of microbial community at 27°C

Counts of the microbial community were performed five days into the second replacement series experiment, as this was the time point during the first experiment at which drastically different survivorship across treatments began to be observed. The only protozoans observed in the treatments were small flagellates; these were counted under a dissecting microscope using standard procedures (Lawler and Morin 1993). Bacteria were fixed in formalin, then enumerated using Acridine Orange Direct Counts (AODC) at 1000x under an epifluorescent microscope (Francisco et al. 1973).
To identify the species of protozoan flagellate found in the treatments, DNA was extracted from pelleted cells from one of the three *Ae. j. japonicus*-only replicates using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). The 18S rDNA gene was amplified in a nested PCR using Eukaryotic-specific primers EukA and EukB (Medlin et al. 1988) for the external PCR and 514F and 1055R (Atkins et al. 2000) for the internal PCR. Internal PCR products of approximately 700bp were cloned using the TopoTA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using the internal primers as forward and reverse sequencing primers. The resulting sequence was then compared to other 18S sequences of known origin in NCBI’s GenBank using a BLASTn search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and top matches from this search (>98% match) were considered good candidates for the taxonomic identification of the unknown flagellate.

**Statistical analysis.**

The proportion of specimens surviving to adulthood (calculated as #emerged/#initial of each species), bounded by zero and one, was arcsine square root transformed to meet assumptions of normality. All other variables met criteria of normality and homogeneity of variances and therefore were not transformed. In the first replacement series experiment, we tested for the effect of the explanatory variables, temperature and species ratio, on the response variables, survival to adulthood and adult female wing length, in separate two-way ANOVAs for each species. Post-hoc Tukey tests were conducted for the factor treatment ratio to uncover differences between groups. In the case of temperature, since there were only two treatments, *t*-tests for independent
samples were performed instead of Tukey tests to examine specific effects of temperature.

For the second replacement series experiment, we performed a series of one-way ANOVAs to determine the effect of species ratio treatment on *Ae. j. japonicus* survival, flagellate counts, and bacterial counts. All statistical analyses were performed using PASW Statistics 18, Release Version 18.0.0 (SPSS, Inc., Chicago, IL).

**Results**

*Replacement series experiments*

In the first experiment, we found that at 27°C *Ae. j. japonicus* larvae only survived to emergence in the lower density *Cx. quinquefasciatus* treatments. All *Ae. j. japonicus* in the three replicates of the 0Q:100J treatments at 27 °C died within 5-7d. In the treatments with *Cx. quinquefasciatus* at 27°C *Ae. j. japonicus* adults emerged from 25Q:75J and 50Q:50J treatments, though there was also no *Ae. j. japonicus* survival in the 75Q:25J treatments (Fig. 1b). Accordingly, an ANOVA found that *Ae. j. japonicus* survival was significantly affected both by temperature and treatment, and marginally affected by their interaction (Table 1.1). In fact, they exhibited significantly lower survival in all treatments at 27°C than at 17°C (7.22% vs. 51.17%, \( t=7.429, \) df=22, \( p<0.001 \)). Conversely, their size did not differ significantly across either temperature or species ratio (Table 1.1).

In contrast, for *Cx. quinquefasciatus* both their survival and size were significantly affected by species ratio (Table 1.1). Post-hoc tests revealed they were significantly smaller at their greatest density (100Q:0J) compared to all other treatments (\( p<0.001 \) for comparison to 25Q:75J and 50Q:50J, and \( p=0.001 \) for 75Q:25J) (Table 1.2).
Similarly, their highest survivorship was in treatments with their lowest density (in 25Q:75J treatments relative to 75Q:25J and 100Q:0J treatments, Tukey HSD, p=0.032 and 0.041, respectively).

In the second replacement series experiment, the pattern observed in the first experiment at 27°C was repeated: all *Ae. j. japonicus* in 0Q:100J replicates died, while some *Ae. j. japonicus* survived in the treatments with low numbers of *Cx. quinquefasciatus*, 25Q:75J and 50Q:50J (Fig. 1.1c). Despite our attempts at standardization the second experiment had greater variability in *Ae. j. japonicus* survival and therefore the effect of species ratio on *Ae. j. japonicus* survival was not significant, though it showed the same trend as previously (Fig. 1.1c, F=3.40, df=3, p=0.074). *Cx. quinquefasciatus* again demonstrated decreasing survival with increasing *Cx. quinquefasciatus* (F=15.006, df=3, p=0.001) (Fig. 1.1c) and this was particularly extreme in the 100Q:0J treatments relative to 25Q:75J (Tukey HSD, p=0.001).

**Analysis of microbial community at 27°C**

Bacterial abundance differed among species ratio treatments (F=16.507, df=4, p<0.001) with the 100J:0Q and 75J:25Q treatments having significantly more bacteria than the three treatments with more *Cx. quinquefasciatus* (Fig. 1.2a). Flagellate abundance also showed a significant effect of species ratio (F=57.845, df=4, p<0.001) where the *Ae. j. japonicus*-only treatment (100J:0Q) had significantly more flagellates than any of the treatments with *Cx. quinquefasciatus* (Fig. 1.2b).

Flagellate DNA was successfully extracted, amplified and sequenced. Two species of flagellates were identified in GenBank BLAST searches: One as *Colpodella sp.* with 88% coverage and 98% identity and one as *Spumella sp.* with 99% coverage and
identity. Based on morphological characters including shape and presence of a yellow plastid, *Spumella sp.* was identified as the abundant species in *Ae. j. japonicus*–only treatments, while *Colpodella* was relatively rare. Sequences of the two flagellates in our experiment have been entered into GenBank with accession #s JX624256 and JX624257.

**Discussion**

The low overall survival of *Ae. j. japonicus* at 27°C is not surprising given that it is a cold-adapted species (Tanaka et al. 1979, Scott 2003) although they have survived in the laboratory at 25°C if the water in the rearing pans is changed daily (Williges et al. 2008) or if provided with a different resource base (oak leaf-infusion instead of rat chow) (Armistead et al. 2008b). Of note, our use of rat chow and lack of water changes did not result in as extreme *Ae. j. japonicus* mortality at 17°C, where they survived in similar numbers to *Cx. quinquefasciatus*. This result confirms that rat chow can be an appropriate food source for *Ae. j. japonicus* and it is the interaction between food resource and high temperature that produces a microbial community detrimental to *Ae. j. japonicus* survival.

Consistent with previous reports for this species (Smith et al. 1995, Agnew et al. 2000) *Cx. quinquefasciatus* was more greatly affected by intraspecific than interspecific competition, exhibiting reduced survival and size in treatments with higher conspecific densities. Their ability to survive overcrowded conditions by altering parameters like body size may help to explain why their larvae are often observed at high densities in the field. In contrast, *Ae. j. japonicus* did not alter their size in response to density, perhaps because their tolerance to cold allows them to utilize containers less likely to be inhabited by other species both spatially and temporally (Bartlett-Healy et al. 2012, Kaufman and Fonseca 2014), and therefore they would not need adaptations to contend with
overcrowding like *Cx. quinquefasciatus*. Evidence from other laboratory experiments with *Ae. j. japonicus* demonstrates that adult size is typically unaffected by density, but instead their development rates are slowed, taking longer to become adults (Armistead et al. 2008a, Alto 2011, Hardstone and Andreadis 2012). *Ae. j. japonicus* are also capable of persisting for months as larvae under cooler temperatures (Scott 2003) so it is possible that they may survive unfavorable environments by slowing their growth until more favorable conditions emerge (e.g. in the case of competitive stress, when the other species stops feeding and pupates). However, developmental rates are also strongly tied to temperature, so they may be unable to utilize this strategy above a certain temperature threshold and instead suffer mortality. While our experiment was not designed to compare development rates, such a possibility merits further research attention as it may help to explain their rapid and extensive expansion across temperate regions.

It is apparent from the bacterial and flagellate counts that *Cx. quinquefasciatus* and *Ae. j. japonicus* affect the microbial community in different ways. The abundant flagellates, identified as *Spumella sp.*, gave the *Ae. j. japonicus*-only treatments a cloudy, yellowish color which was not observed in treatments with *Cx. quinquefasciatus* or in any of the low temperature treatments. *Spumella* are Chrysomonads, called “golden algae” due to the presence of the pigment fucoxanthin, which gives them a yellowish-brown color (Pearson 1995). They are small, heterotrophic, unicellular protists commonly found in freshwater aquatic environments all over the world (Boenigk et al. 2005, Boenigk et al. 2006) and they form cysts to survive harsh environmental conditions (Findenig et al. 2010). We hypothesize either that *Cx. quinquefasciatus* is able to consume *Spumella*, whereas *Ae. j. japonicus* cannot, or it consumes them in much larger numbers than *Ae. j.
japonicus, thereby decreasing flagellate populations (Fig. 1.3a & b). Indeed, Aedes and Culex species are known to differ in their feeding behavior: Aedes primarily browse and scrape along the sides and bottom of containers while Culex filter feed on particles in the water column (Merritt et al. 1992, Kaufman et al. 2001, Yee et al. 2004). There is evidence that Spumella prefer to feed in the water column (Lavrentyev et al. 1997) so Cx. quinquefasciatus may be more likely to encounter them than Ae. j. japonicus.

In summary, we postulate two alternative hypotheses to explain the high Ae. j. japonicus mortality in the presence of large numbers of Spumella. First, it is possible that Spumella harm Ae. j. japonicus directly, perhaps through production of a toxin or byproduct deadly to them (Fig. 1.3a). Some strains of Spumella are known to be toxic to zooplankton and cause mortality only when present in large enough numbers (Boenigk and Stadler 2004). An alternative hypothesis is that Spumella harm Ae. j. japonicus indirectly, by altering the microbial community in a way that makes conditions unfavorable for Ae. j. japonicus (Fig. 1.3b). Since we did not see a decrease in total bacteria in treatments with high numbers of Spumella this change would have to be in the composition of the microbial community, shifting it away from those species which are not harmful to Ae. j. japonicus or provide food for them (“Ae. j. japonicus-friendly bacteria”) and towards harmful or inedible species. Supporting this hypothesis, there is evidence that Spumella will alter bacterial communities (Bell et al. 2010) and that certain types of bacteria are harmful to mosquitoes, for example, Pseudomonas aeruginosa contains toxic lipopolysaccharides that slow the growth rate of Cx. tarsalis (Peck and Walton 2006). Interestingly, high P. aeruginosa counts in bodies of water are often associated with sewage pollution (de Vicente et al. 1991) and perhaps as a consequence
of its adaptation to polluted environments, *Cx. quinquefasciatus* is unaffected by their toxins (Peck and Walton 2006)

A final question posits why the interaction between *Cx. quinquefasciatus* and *Ae. j. japonicus* appears to change with increasing *Cx. quinquefasciatus* density at 27°C.

Levine (1976) showed that the outcome of an interaction between two competing species can be positive so long as the benefit gained by suppression of a third species outweighs the direct negative consequences of competition (Brooker et al. 2008). Therefore, it is possible that the interaction between *Cx. quinquefasciatus* and *Ae. j. japonicus* could be positive when the benefit gained by *Ae. j. japonicus* from suppression of *Spumella* outweighs the effects of competition with *Cx. quinquefasciatus*, i.e. when *Spumella* numbers are high and *Cx. quinquefasciatus* numbers are low. Density has a well documented effect on altering the sign of interactions between plant species that share pollinators (Thomson 1981, Moeller 2004, Ghazoul 2006); although in animal communities, density has only been shown to affect the strength of the interaction, not the sign (Irving and Bertness 2009, Bishop et al. 2012).

Our results raise the interesting question of whether *Ae. j. japonicus* (an unlikely invader of Hawaii, given its geographic distribution and temperature tolerances) may have been facilitated in its establishment there by the presence of *Cx. quinquefasciatus*. *Ae. j. japonicus* was first collected at low elevations along Hawaii’s coast, where temperatures are warmer, and only later spread up to cooler higher elevations (Larish and Savage 2005, Larish et al. 2010). Females of both species have been captured in gravid traps baited with leaf infusion at both low and high elevations (Larish et al. 2010) indicating that they are attracted to similar oviposition cues and likely lay eggs in the
same containers. Larvae of the two species have been collected from the same containers at both low and high elevations (Egizi AE personal observation) and flagellates in the genus *Spumella* have been identified in Hawaii (Boenigk et al. 2006) indicating that while our results may have some relevance, field experiments and abundance surveys conducted in Hawaii are required to consider this hypothesis. Another, more general implication of our results is that pollution-tolerant species may be able to ameliorate environments, making them more suitable for less tolerant species. Just as negative interactions can decrease the realized niche of a species, positive interactions can increase it (Bruno et al. 2003, Rodriguez-Cabal et al. 2012, Stachowicz 2012). If mosquitoes are capable of increasing the geographic range and/or microhabitat usage of other mosquitoes, this could result in the establishment of new diseases or increased transmission efficiency of existing ones, with devastating impacts on native wildlife and humans alike.

The interactions we observed in our experiments are indirect and likely mediated by multiple species across the food web, with interesting implications for the ecology of these container habitats. Future experiments to elucidate the mechanism should focus on identifying how each mosquito species responds to the *Spumella* flagellates, how the flagellates affect the bacterial community, and how any resulting changes in the bacterial community affect the mosquitoes, in order to fully grasp the complex interactions occurring in these artificial containers. It is important to study the interactions of mosquito larvae because their outcome will directly influence adult population parameters such as abundance and longevity, and in turn, their ability to vector disease. It is also important to understand how these interactions may change across contexts, for
example, in different climates or with different microbial assemblages. As climate change and globalization continue to produce new ecological communities, these studies will be essential for predicting changes in ecosystem processes that affect disease transmission.

Acknowledgements

The authors thank Linda Larish and Dr. Dennis LaPointe for answering questions and providing assistance regarding the establishment of *Ae. j. japonicus* on Hawaii. We thank Linda McCuiston for providing *Ae. j. japonicus* and *Cx. quinquefasciatus* eggs, Kelly Pniewski and Laran Kaplan for laboratory assistance, and Cara Fallaice for helpful comments. We also owe a debt of gratitude to the editor, Dr. Michael T. Monaghan, and to two anonymous reviewers for comments that substantially improved the manuscript. This work was funded by Rutgers start-up funds to D. M. Fonseca, and a Rutgers Ecology and Evolution Graduate Program Small Grant, and Northeastern Mosquito Control Association’s Jobbins Scholarship, to A. Egizi. Experiments comply with current U.S. laws.
Table 1.1 Results of two way ANOVAs examining the effects of temperature and treatment on *Cx. quinquefasciatus* and *Ae. j. japonicus* survival and size. Survival = proportion of larvae that survived to adult emergence, and size = mean adult female wing length.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Factor</th>
<th><em>Cx. quinquefasciatus</em></th>
<th></th>
<th></th>
<th><em>Ae. j. japonicus</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>df</td>
<td>P</td>
<td>F</td>
<td>df</td>
<td>P</td>
</tr>
<tr>
<td>Survival</td>
<td>Temperature</td>
<td>0.077</td>
<td>1</td>
<td>0.785</td>
<td>88.29</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Species ratio</td>
<td>4.152</td>
<td>3</td>
<td>0.024</td>
<td>3.24</td>
<td>3</td>
<td>0.0499</td>
</tr>
<tr>
<td></td>
<td>Temperature x Ratio</td>
<td>0.956</td>
<td>3</td>
<td>0.437</td>
<td>3.16</td>
<td>3</td>
<td>0.0537</td>
</tr>
<tr>
<td>Size</td>
<td>Temperature</td>
<td>3.444</td>
<td>1</td>
<td>0.082</td>
<td>0.050</td>
<td>1</td>
<td>0.828</td>
</tr>
<tr>
<td></td>
<td>Species ratio</td>
<td>16.420</td>
<td>3</td>
<td>&lt;0.001</td>
<td>1.228</td>
<td>3</td>
<td>0.346</td>
</tr>
<tr>
<td></td>
<td>Temperature x Ratio</td>
<td>1.493</td>
<td>3</td>
<td>0.254</td>
<td>0.374</td>
<td>1</td>
<td>0.553</td>
</tr>
</tbody>
</table>
Table 1.2 *Cx. quinquefasciatus* and *Ae. j. japonicus* mean adult female wing length.

Dashes represent treatments from which no adults of that species emerged. Standard error is across individuals.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Ratio Treatment</th>
<th>Cx. quinquefasciatus Mean female wing length ± SE</th>
<th>Ae. j. japonicus Mean female wing length ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>0Q:100J</td>
<td>--</td>
<td>3.56 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>25Q:75J</td>
<td>3.32 ± 0.056</td>
<td>3.47 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>50Q:50J</td>
<td>3.32 ± 0.036</td>
<td>3.49 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>75Q:25J</td>
<td>3.32 ± 0.033</td>
<td>3.60 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>100Q:0J</td>
<td>3.02 ± 0.034</td>
<td>--</td>
</tr>
<tr>
<td>27</td>
<td>0Q:100J</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>25Q:75J</td>
<td>3.34 ± 0.030</td>
<td>3.39 ± 0.035</td>
</tr>
<tr>
<td></td>
<td>50Q:50J</td>
<td>3.32 ± 0.026</td>
<td>3.56 ± 0.042</td>
</tr>
<tr>
<td></td>
<td>75Q:25J</td>
<td>3.16 ± 0.030</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>100Q:0J</td>
<td>2.86 ± 0.027</td>
<td>--</td>
</tr>
</tbody>
</table>
Fig. 1.1 Percent adult emergence across temperatures and treatments at (a) 17°C and (b) 27°C in first replacement series experiment, and (c) at 27°C in second replacement series experiment. Blue diamonds represent *Aedes j. japonicus* and brown circles represent *Culex quinquefasciatus*. Error bars are one standard error of the mean across replicates.
Fig. 1.2 Direct counts of (a) bacteria and (b) flagellates across species ratio treatments. Error bars are two standard errors across replicates. Uppercase letters indicate significant differences among ratio treatments (P<0.05) from Tukey post-hoc tests following one-way ANOVAs.
Fig. 1.3 Interaction map displaying two hypothesized mechanisms by which Cx. quinquefasciatus may affect Ae. j. japonicus survival, mediated through Spumella: (a) Spumella flagellates have a direct negative effect on Ae. j. japonicus, such as through toxicity; (b) Spumella flagellates affect Ae. j. japonicus indirectly through manipulation of the bacterial community. Solid lines indicate a direct interaction and dotted lines an indirect interaction.
Chapter 2:

Ecological limits can obscure expansion history: patterns of genetic diversity in a temperate mosquito in Hawaii

Abstract

Because biological invasions can be swift and are rarely examined immediately and/or followed over time, spatial genetic diversity analyses grounded in a well-developed body of theory are often used to reconstruct historical patterns of expansion. Unfortunately, the role of selection in shaping and potentially disrupting such reconstructions has seldom been examined. The mosquito *Aedes japonicus japonicus* is a temperate, cold-adapted species native to northern Japan that has recently established populations on the island of Hawaii. We used variation at seven microsatellite loci and one mitochondrial locus to examine Hawaiian populations collected in 2004, shortly after its first detection, and then in 2010-2011. Samples were collected along an elevational/temperature gradient, ranging from sea level to 1200 m. Specimens collected near sea level in 2004 from the earliest detected population exhibited high genetic diversity. Contrary to expectations that diversity would decrease outward from the point of introduction, in 2010-2011 high elevation populations had the greatest genetic diversity, while low elevation populations (including those with high diversity in 2004) now had lower diversity and were significantly differentiated from each other, suggesting severe bottlenecks. We hypothesize that differential survival across temperatures at high vs. low elevations has subverted the expected genetic signature of an expanding population.
Introduction

Due to the increasing prevalence of invasive species and climate-induced range shifts, it is important to study the processes involved in species range expansions. Specifically, the genetic processes that accompany such expansions can have important consequences for the evolutionary potential of the expanding species. Subsets of individuals that move beyond the range boundary are predicted to experience a decline in genetic diversity (loss of alleles) known as the founder effect (Mayr 1942, Nei et al. 1975). Sequential founder effects along the axis of an expansion will cause further decreases in genetic diversity and increased differentiation (Austerlitz et al. 1997). This pattern persists longer when rates of gene flow and population growth are low (Austerlitz et al. 1997) and when dispersal is local and restricted to populations of the same age (Le Corre and Kremer 1998). Wegmann et al. (2006) incorporated environmental heterogeneity into spatial expansion models, as defined by demes with different carrying capacities, and found that heterogeneity increases genetic differentiation among populations and decreases diversity within populations. They also predicted that metapopulations with larger mean carrying capacities are more robust to the effects of environmental heterogeneity (Wegmann et al. 2006).

These models approximate the conditions likely experienced by expanding species but fail to take into consideration the effects of selection. Furthermore, despite extensive theoretical groundwork (Excoffier et al. 2008), empirical studies on the genetics of range expansion remain rare. Most studies examine genetic patterns over large spatial scales, such as a species’ entire range (Eckert et al. 2008) and/or look for a signature of historical expansion, such as postglacial re-colonization (Hewitt 2000). Unfortunately, older expansions have had time for gene flow between the leading edge of
the expansion and range center to obscure any spatial signal, necessitating theory to distinguish older spatial expansions from demographic expansion alone (Ray et al. 2003, Excoffier 2004). Although recent expansions like those of invasive species may not meet key assumptions of population genetic models such as mutation-drift equilibrium, complicating interpretation of their genetic patterns (Fitzpatrick et al. 2012), studies of invasive species have supported theoretical predictions (high genetic diversity at the point of origin, decreasing outward) (Amsellem et al. 2000, Estoup et al. 2004, Herborg et al. 2007, Short and Petren 2011, Schulte et al. 2013). We hypothesize, however, that spatially heterogeneous selection could produce spatial genetic patterns alternative to those expected from expansion alone. We used the arrival of the mosquito *Aedes japonicus japonicus* on Hawaii, i.e. the recent spread of an invasive species into an atypical environment, and our ability to make comparisons over time, to test some of the basic predictions of spatial genetic models in species invasions.

*Aedes j. japonicus* is a cold-adapted species that lays diapausing eggs to survive the winter (Tanaka et al. 1979). While native to southern Siberia, northern Japan and Korea (Tanaka et al. 1979), this species has recently expanded into the northern US, Canada, and northern Europe (Peyton et al. 1999, Roppo et al. 2004, Thielman and Hunter 2006, Schaffner et al. 2009, Kaufman and Fonseca 2014). Surprisingly, despite being largely restricted to colder latitudes elsewhere in the world, it established on the island of Hawaii in 2003 (Larish and Savage 2005). Hawaii is the youngest and largest of the Hawaiian Islands and its topography contains steep elevational gradients due to its two large volcanoes. Due to trade winds, the eastern side of the island receives heavy rainfall and is lush with rainforest, while the western side of the island is dry and desert-
like. *Aedes j. japonicus* was first collected in Laupahoehoe, a town on the northeastern coast (Larish and Savage 2005), and most likely arrived via ship or airplane traffic into the nearby town of Hilo (Fig. 2.1). This mosquito has subsequently spread across much of the island, particularly throughout higher elevations on the eastern side, though remaining limited to just the one island (Larish et al. 2010).

Elevation is known to affect genetic patterns in frogs (Funk et al. 2005), salamanders (Spear et al. 2005), other insects (Liebherr 1986, Schiffer et al. 2007, Lozier et al. 2011), and plants (Ohsawa and Ide 2008), usually due to changes in temperature, air pressure, or lack of gene flow from nearby populations. Hawaii’s elevational gradient is accompanied by a steep change in temperature: the mean August temperature in Hilo (near location C, 135 m, Fig. 2.1) was 24.6ºC for the period 1949-2000, while in Volcanoes National Park (location G, 1200 m, Fig. 2.1) it was 17.6ºC (Hawaii State Climate Office, http://www.soest.hawaii.edu/MET/Hsco/temp.htm). The temperature/elevation gradient on Hawaii has influenced spatial genetic patterns in another invasive mosquito, *Culex quinquefasciatus*, a tropical species introduced to Hawaii in the 1820’s. Keyghobadi et al. (2006) showed that year-round populations of *Cx. quinquefasciatus* on Hawaii are mainly restricted to warm, lower elevations (0-305 m), with only temporary summer populations at cool, higher elevations (900-1305 m). As *Ae. j. japonicus* is a temperate species, we anticipated that it might instead have more stable populations at (cooler) high elevations. Indeed, a laboratory experiment examining *Ae. j. japonicus* larval development at temperatures ranging from 10-40ºC found the highest survival occurred at 16ºC (Scott 2003), and another experiment comparing 17ºC and 27ºC demonstrated better *Ae. j. japonicus* survival at 17ºC (Egizi et al. 2014).
Our specific questions were as follows: (1) Are populations of *Ae. j. japonicus* genetically structured across elevation (and, by extension, temperature)? (2) Do genetic patterns of *Ae. j. japonicus* in Hawaii match theoretical expectations of classic spatial expansion models?

**Methods**

**Sampling in Hawaii**

In August 2010, samples were collected from six different sites on the island of Hawaii, including sites at different elevations (Table 2.1). In August 2011, more specimens were collected from one low elevation site, as well as two additional sites on different parts of the island. This resulted in a total of 244 specimens from eight different sites (Fig. 2.1, Table 2.1). There was one additional collection site at mid elevation (Cem11), for which we were only able to collect one *Ae. j. japonicus*, and this sample was genotyped for the mitochondrial locus but omitted from the microsatellite analyses. Although we attempted to collect similar numbers of larvae from all sites, we found lower numbers at most low elevation sites. Adult *Ae. j. japonicus* were not successfully captured using either CDC light traps (Bioquip Products, Rancho Domingo, CA) or BioGents Sentinel traps (Biogents, AG, Regensburg, Germany), so specimens were mainly collected as larvae. The only exception was the Volcano site, where adult specimens were obtained from Hawaiian Volcanoes-USGS, collected originally as larvae from habitat near the USGS field station and reared to adulthood. Although females of several *Aedes* species spread their eggs across multiple containers (Reiter 2007, Fonseca et al. 2014), mosquito larvae collected from the same container may still be siblings. To avoid oversampling within families, we sampled multiple containers within each site,
with the exception of the Scenic Rt site, where we only found one container with *Ae. j. japonicus* (a very large, water-filled palm leaf). Other types of containers where we collected *Ae. j. japonicus* include tires, cemetery vases, rock pools, and Hapu’u cavities (holes created in downed Hawaiian tree ferns, *Cibotium chamissoi*, by feral pigs) (LaPointe 2006). Specimens were collected using a 30 ml plastic pipette, separated and identified inside a white plastic tray, and then transferred into 2 ml cryovials with 100% ethanol until extraction.

We also analyzed samples from a 2004 collection of *Ae. j. japonicus* in Laupahoehoe, a low elevation site (Table 2.1), to examine temporal patterns of change. To elucidate a putative origin, we also compared our data with results from the mainland US introduction of *Ae. j. japonicus* (Fonseca et al. 2010). The eastern US experienced two separate and genetically distinct introductions of *Ae. j. japonicus*, one into New York and New Jersey (NYNJ) and one into Pennsylvania (PA) (Fonseca et al. 2010), so we included representative samples from both areas in our analysis, as well as representative samples from the Japanese cities of Tokyo and Kyoto (Fonseca et al., unpublished data).

*Microsatellite genotyping*

DNA was extracted from all Hawaiian collections using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). All samples were assayed for a panel of seven microsatellite loci (Widdel et al. 2005), using the modified primer OJ5R3 (instead of OJ5R) to reduce null alleles following the protocols in Fonseca et al. (2010). PCR products were sized in an ABI 3130XL Genetic Analyzer 16 Capillary machine (Applied Biosystems, Foster City, CA). Alleles were scored with Gene Mapper 3.5 (Applied
Biosystems, Foster City, CA), using bins optimized on worldwide populations of *Ae. j. japonicus*.

**Mitochondrial sequencing**

We amplified 420 bp of the mitochondrial sodium dehydrogenase subunit 4 (ND4) locus from ten specimens in each population, using the same protocol as Fonseca et al. (2001), but with a modified reverse primer, ND4R1X: 5’-TGATTGCCTAAGGCTCATGT-3’ that is a better match to *Ae. j. japonicus* sequences. The ND4 locus was chosen because haplotype data were already available for many worldwide populations and this locus is known to be informative for genetic patterns across the mainland US and Europe (Fonseca et al. 2001, Fonseca et al. 2010, Zielke et al. 2014). PCR products were cleaned with ExoSap-IT (Affymetrix, Santa Clara, CA), cycle sequenced, and then sized on an ABI 3730XL Genetic Analyzer 96 capillary machine (Applied Biosystems, Foster City, CA). The sequences were assembled and cleaned in Sequencher 4.10.1 (GeneCodes Co., Ann Arbor, MI).

**Population genetic analyses**

To examine genetic diversity patterns at different elevations, we used the program FSTAT 1.2 (Goudet 1995) to calculate allelic richness (A_R) which was standardized to the lowest population size (N = 13) using rarefaction (Leberg 2002). We also calculated Shannon’s information index (I), mean number of alleles (N_a), observed heterozygosity (H_o), and unbiased expected heterozygosity (uH_e) for each population in GenAlEx 6.501 (Peakall and Smouse 2012). Shannon’s information index, often used in community composition studies, is a measure of diversity that includes the frequency of each species in addition to the total number, and can be applied to calculate genetic diversity as well
(Sherwin et al. 2006, Sherwin 2010). Three diversity measures ($A_R$, $I$ and $uH_e$) were regressed against elevation in a linear model using the statistical software program SPSS (Release Version 18.0.0, SPSS, Inc., Chicago, IL). To test for the effect of elevation on genetic differentiation, we calculated population-specific $F_{ST}$ values using the Bayesian $F$-model implemented in GESTE 2.0 (Foll and Gaggiotti 2006), under default parameters. In this program population-specific $F_{ST}$ values are derived from the comparison between each population and the total gene pool, so they act as a measure of how much each population has differentiated. This approach takes into consideration that populations often differ in their effective population sizes and migration rates, therefore population-specific $F_{STS}$ may be a more informative descriptor of genetic structure than pairwise comparisons (Foll and Gaggiotti 2010). Linear regression models for the effects of elevation on population-specific $F_{ST}$ values were estimated in SPSS (Release Version 18.0.0, SPSS, Inc., Chicago, IL). $F_{IS}$ values were calculated in GenePop v.4.1.2 (Raymond and Rousset 1995).

To uncover genetic patterns resulting from factors other than elevation, we examined population differentiation across all Hawaiian populations. We determined population structure based on individual multi-locus signatures, a Bayesian approach, in STRUCTURE (Pritchard et al. 2000) using the model with admixture. The optimal number of clusters ($K$) was determined using the method of Evanno et al. (2005). GenAlEx software v.6.501 (Peakall and Smouse 2012) was used to calculate Nei’s unbiased index of divergence, which formed the distance matrix plotted with non-metric multidimensional scaling (NMDS) using the “metaMDS” routine in the R package vegan (Oksanen et al. 2013, R Core Team 2013). Pairwise $F_{ST}$ values were calculated and
checked for significance in FSTAT 1.2 (Goudet 1995) and then tested against geographic
distance in a Mantel test in GenAlEx (Peakall and Smouse 2006). GenAlEx was also
used to perform an analysis of molecular variance (AMOVA) on $F_{ST}$, examining
variation within and among Hawaiian populations and calculating $F'_{ST}$ (Peakall and
Smouse 2012). $F'_{ST}$ is calculated by dividing the observed $F_{ST}$ by the maximum possible
$F_{ST}$ for two populations that share no alleles (Hedrick 2005). This standardization aids in
the interpretation of among population differentiation using highly polymorphic markers
such as microsatellites where the maximum value of $F_{ST}$ is not 1 (Hedrick 2005).

Results

Hawaiian populations

Multiple measures of genetic diversity were positively correlated with elevation
among the 2010-2011 populations. Standardized $A_R$ ($r = 0.81, P < 0.01$) (Fig. 2.2a) and
Shannon’s $I$ ($r = 0.77, P < 0.02$) (Table 2.1) were strongly and significantly correlated,
while $H_e$ was less strongly correlated and the fit was not significant ($r = 0.66, P = 0.054$
(Table 2.1). Population differentiation as measured by population-specific $F_{ST}$ values was
negatively correlated with elevation ($r = -0.72, P < 0.03$ (Fig. 2.2b). $F_{IS}$ values revealed
most populations to have a slight heterozygote excess (between $-0.05$ and $0.004$) with the
exceptions being Rainbow Falls 2010, which had a larger excess ($-0.110$), and
Mamalahoa, which had a large deficit ($0.195$) (Table 2.1). Rainbow Falls is significantly
different from high elevation populations in 2011, but not 2010, and the large
heterozygote excess further suggests a recent influx of migrants in 2010 that had become
panmictic by 2011.
While the multilocus Bayesian analysis in STRUCTURE failed to detect any structure within Hawaii, the NMDS plot of Nei’s unbiased distances (stress = 0.059, Fig. 2.3) agreed with the pattern of genetic differentiation observed from the population-specific $F_{ST}$’s. Low elevation populations are more distinct from each other and from the original introduction than the mid or high elevation populations, which cluster with the original introduction (Fig. 2.3). Similarly, pairwise $F_{ST}$ values (Table 2.2) indicated that low elevation populations are significantly different from each other while high elevation populations are quite similar to each other. A Mantel test of the association between genetic similarity and geographic distance was not significant ($R^2 = 0.0004, P = 0.661$) indicating that linear geographic distance is not as important as elevation as a factor influencing Hawaiian populations. An AMOVA test found 96% within-population variation and 4% between-populations (an overall $F_{ST}$ of 0.04, with $P = 0.001$ for 999 permutations). While this seems low, the maximum attainable $F_{ST}$ was only 0.425, giving $F'_{ST} = 0.10$ (meaning that the observed $F_{ST}$ value is 10% of its maximum attainable value).

We only identified two ND4 haplotypes in Hawaiian Ae. j. japonicus: H1 and H9 (N = 81). The majority of samples were H1, and H9 was only found in two individuals out of the 81: one from the Volcano population and one from the mid-elevation population with only one individual, Cem11. These two ND4 haplotypes were originally characteristic of the two separate introductions of this species to the US east coast, H1 in NY/NJ and H9 in PA (Fonseca et al. 2010). Of note, both are also common in populations of Ae. j. japonicus in Japan’s large cities (Fonseca et al. 2001).
Comparison of Hawaii with continental US and Japan

In the NMDS plot of Nei’s unbiased distances, NYNJ and Japan appear closer to Hawaiian specimens than does PA (Fig. 2.3). A STRUCTURE analysis (not shown) distinguishes the Hawaiian specimens from all potential source populations (PA, Japan, NYNJ) with $K = 2$ as the most likely number of separate gene pools (Evanno et al. 2005). Subsequent analyses in STRUCTURE excluding the PA specimens and/or including only a subset of Hawaiian populations consistently identified Hawaii as a distinct cluster. Pairwise $F_{ST}$ values are large and significant between all Hawaii and non-Hawaii populations, though the values are larger between HI and PA (ranging from 0.21 to 0.37) than they are between HI and NYNJ (ranging from 0.04 to 0.22) or HI and Japan (ranging from 0.04 to 0.17) (Table 2.2).

The haplotypes found in Hawaii, H9 and H1, suggest that a mix of the two original US introductions, NYNJ and PA, could have been introduced into Hawaii (Fonseca et al. 2001, 2010). However, H1 and H9 are also found in Japan (Fonseca et al. 2001) and there are two microsatellite alleles present in Hawaii (154 at OJ187, and 187 at OJ338) that are in Japan but were not detected in any part of the continental US introduction (Fonseca et al., unpublished data). These alleles could still be present in US populations at very low frequencies, of course, so this does not preclude a US origin. Based on our results, we cannot assess definitively whether the Hawaiian introduction came as a secondary introduction from the eastern US or directly from Japan, but it seems unlikely there was more than a single event.
Discussion

Elevation or its correlate, temperature, appears to be the most significant landscape factor affecting Hawaiian *Ae. j. japonicus*. There was greater genetic differentiation among and less variation within low elevation populations when compared to high elevation populations, and both allelic richness and Shannon’s information index were strongly positively correlated with elevation. While expected heterozygosity was not significantly related to elevation, this may be due to its greater sensitivity to population growth rate (following bottlenecks) than measures like allelic richness (Nei et al. 1975). Most studies reporting elevational effects (Liebherr 1986, Funk et al. 2005, Spear et al. 2005, Schiffer et al. 2007, Ohsawa and Ide 2008, Lozier et al. 2011) found lower genetic variation and increased differentiation at higher elevations, indicating these populations are less stable and more isolated as a result of increased physiological demands at high altitudes. We observed the opposite: lower genetic variation and increased differentiation at low elevations, a rare pattern, but one also detected by Wen & Hsiao (2001) in populations of the Taiwan lily. Wen & Hsiao (2001) hypothesized that this was due to the detrimental effects of human populations at low elevations, such as unrestricted collecting and increased disturbance. Likewise in Hawaii lower elevations have the highest human population densities, and collection sites tended to be along roads. However, it is unlikely that human population density is having a negative effect on *Ae. j. japonicus*, as the mid elevation sites were also alongside roads but did not exhibit significantly lower genetic diversity than high elevation sites.

A more likely hypothesis is that *Ae. j. japonicus* populations are affected by the temperature differences between elevations. Hawaii’s lower elevations are likely too
warm for optimal larval development of *Ae. j. japonicus* and this appears to have a profound effect on both their distribution and genetic architecture. In fact, during our collections they were more difficult to find at low elevations, requiring more intensive sampling effort. This observation agrees with the genetic evidence, as the genetic signature of low elevation populations suggests repeated bottlenecks and patchier populations at low elevation. The tropical mosquito *Cx. quinquefasciatus* has more stable populations at low elevations in Hawaii and can only sustain temporary populations in colder high elevation habitat (Keyghobadi et al. 2006). It is probable that the reverse situation is occurring with the temperate *Ae. j. japonicus* where this species re-colonizes warmer low elevations from cooler high elevations. This directional gene flow in addition to their recent common genetic ancestry may account for the lack of pronounced allele frequency differences between low and high elevation populations.

The observed loss of genetic diversity at low elevations could be related to a selective sweep in response to selection on temperature tolerance. Invasive insects are capable of rapid evolution: an invasive fly (Huey et al. 2000, Mestres et al. 2004) and invasive mosquito (Urbanski et al. 2012) both developed latitudinal clines in phenotypic traits less than 50 years post introduction and despite presumably small founding populations. Other groups of invasive taxa have proven capable of responding to novel temperature requirements; for example the European rabbit, introduced to Australia in 1859, appears to have developed thermally adaptive differences in body size since that initial introduction (Williams and Moore 1989). Barnyard grass is believed to have invaded Canada from southern parts of North America in the last 400 years (Roy et al. 2000) and populations from the extremes of its range show clear signs of temperature
adaptation: northern plants do not survive the summer in the south, and southern plants do not flower in the north (Potvin et al. 1986). Future studies could examine whether populations of *Ae. j. japonicus* in Hawaii have adapted to warmer temperatures, as this could herald its spread in more tropical areas in addition to its continued expansion throughout the world’s temperate zones.

An interesting outcome of our study was a pattern of genetic diversity in a recently expanded population that contradicts the predictions of spatial expansion models. The first propagules must have entered Hawaii at low elevations, as this is where all points of entry (airports, ship docks, etc.) are located, and that is where they were first detected (Larish and Savage 2005). This means that *Ae. j. japonicus* colonized higher elevations from low elevation source populations, and did so within 1-2 years of their first discovery (Larish et al. 2010). By 2010-2011, however, there was no evidence of a decline in genetic diversity along the presumed axis of their expansion, as theoretical models predict. One explanation for the discrepancy may be that the assumptions of these models are not often met in real world scenarios, for example, a homogeneous environment that is selectively neutral (e.g., Austerlitz et al. 1997). If as discussed earlier, *Ae. j. japonicus* survive in much greater numbers at high elevations, they would be able to maintain larger populations, and population growth can counter founder effects (Nei et al. 1975). Over time, as low elevation populations undergo repeated bottlenecks and experience low population sizes, their genetic diversity would decrease relative to that of high elevations, which would be relatively unchanged. Thus climate effects, if operating strongly enough, could quickly wipe out the genetic signal of expansion in favor of the pattern we observed.
We examined an unusual situation: a temperate mosquito introduced into a climate essentially different from its native environment, which led to patterns of genetic diversity and differentiation that do not match predictions by spatial expansion models. Without prior knowledge of where *Ae. j. japonicus* was first collected, and where the points of entry to the island are, we may have concluded that they were introduced into high elevation. Instead we found evidence that selection and rapid differentiation in response to a novel climate can complicate inferences about past demography and challenge theoretical predictions. We therefore encourage researchers to exercise caution when drawing inferences from the genetic structure of invasive species both because they are “in flux,” which may violate the assumptions of genetic models (Fitzpatrick et al. 2012), and because selection is rarely included in such models.

**Acknowledgements**

The authors thank Linda Larish for answering questions about *Ae. j. japonicus* in Hawaii and for generously providing the 2004 specimens; Dr. Dennis LaPointe for answering questions, providing the Volcano specimens and assisting with access to Tree Planting populations; Dr. Jiawu Xu for developing the new ND4 primer; Dana C. Price for assistance in collecting specimens; and Dr. Peter Smouse and Dr. James A. Fordyce, for excellent comments on earlier drafts of this manuscript. This work was funded by a Rutgers Graduate School Pre-Dissertation Travel Award and Rutgers Ecology and Evolution Buell Award to A. Egizi, and by USDA Hatch Grant #NJ08194, NE-1043 Multistate funds, and start-up funds to D. M. Fonseca.
Table 2.1 Hawaiian *Ae. j. japonicus* samples used in microsatellite analysis. Population name is that of the closest town or landmark, Symbol refers to notation in Fig. 2.1, Year = year specimens were collected, N = sample size, \( N_a \) = mean number of alleles across loci, I = Shannon Information Index, \( H_o \) = observed heterozygosity, \( uH_e \) = unbiased expected heterozygosity, and \( F_{IS} \) = inbreeding coefficient.

<table>
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<th>Population</th>
<th>Symbol</th>
<th>Year</th>
<th>Elevation (m)</th>
<th>Latitude</th>
<th>Longitude</th>
<th>N</th>
<th>( N_a )</th>
<th>I</th>
<th>( H_o )</th>
<th>( uH_e )</th>
<th>( F_{IS} )</th>
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<td>19.8047</td>
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<td>0.483</td>
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<td>C</td>
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<td>19.7191</td>
<td>-155.1104</td>
<td>20</td>
<td>3.57</td>
<td>0.924</td>
<td>0.593</td>
<td>0.534</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.931</td>
<td>0.565</td>
<td>0.544</td>
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<tr>
<td>Laupahoeoe</td>
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<td>136.55</td>
<td>19.9821</td>
<td>-155.2325</td>
<td>28</td>
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<td>30</td>
<td>4.14</td>
<td>1.085</td>
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<td>0.609</td>
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<td>4.14</td>
<td>1.040</td>
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<td>0.568</td>
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<tr>
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<td></td>
<td></td>
<td>0.625</td>
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Table 2.2 Pairwise $F_{ST}$ values for the Hawaiian *Ae. j. japonicus* populations. Boxes surround pairwise comparisons between (from left to right) low, mid, and high elevation populations. $F_{ST}$ values marked with a star (*) are significant in FSTAT after 1560 permutations at $P = 0.000641$ (adjusted for multiple comparisons). Values given as 0.00 are less than 0.01.

<table>
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<th>C'11</th>
<th>A'11</th>
<th>D'10</th>
<th>H'11</th>
<th>F'10</th>
<th>E'10</th>
<th>G'10</th>
<th>A'04</th>
<th>PA</th>
<th>NYNJ</th>
<th>Japan</th>
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<td>0.01</td>
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<td>0.21*</td>
<td>0.21*</td>
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<td>0.15*</td>
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Fig. 2.1 Locations on Hawaii (the Big Island) where *Ae. j. japonicus* larvae were collected, and elevational grouping of the locations. Red = low elevation (0-200m), brown = mid elevation (200-600m) and blue = high elevation (600+m). The yellow star indicates the location of *Ae. j. japonicus*’ first collection on Hawaii, Laupahoehoe.
Fig. 2.2 (a) Relationship between mean allelic richness ($A_R$) and elevation. (b) Relationship between population-specific $F_{ST}$ values and elevation. Both relationships are significant (see text).
**Fig. 2.3** Non-metric multidimensional scaling plot of Nei’s Unbiased distances for the 8 Hawaiian populations, NY/NJ, PA, Japan, and initial introduction (star). Numbers represent year of collection.
Chapter 3:

Landscape genetics reveals historical and current drivers of genetic structure in an invasive mosquito

Abstract

Human-aided translocations of species are occurring at an unprecedented rate leading to a recent increase in the number of invasive mosquitoes. Because mosquitoes are potential disease vectors to humans, livestock, and wildlife, it is especially important to understand the main drivers and deterrents underlying their expansion. They are also an excellent model system for biological invasions as there are often extensive and timely surveillance data available. Earlier work revealed that the most recent invasive mosquito, *Aedes japonicus japonicus*, originally from northeast Asia, was introduced twice into the northeastern US. Spatial analysis also demonstrated extensive post-introduction mixing of the two genetically divergent populations. We performed a high-resolution landscape genetic analysis of 461 specimens obtained from a comprehensive surveillance campaign in Virginia, a state south of the original introductions. Virginia’s geography includes colder areas in the Appalachian Mountains that experience temperatures suitable for this temperate mosquito species, as well as much warmer areas near the coast. All specimens were genotyped at seven pre-optimized microsatellite loci and one mitochondrial locus. We found evidence that the expansion of *Ae. j. japonicus* into and across Virginia has primarily involved long distance dispersal events associated with human-aided transportation along roads, coupled with very localized adult diffusive movements. Importantly, our analyses revealed an asymmetrical distribution across elevation of specimens with nuclear genotypes and associated mitotypes matching those of the two original introductions. Significant genetic differentiation associated with elevation
despite extensive gene flow across the state suggests temperature-driven selection and potentially local adaptation in this invasive mosquito.

**Introduction**

It is widely recognized that the current rate and geographic extent of biotic invasions are linked to human activity (Meyerson and Mooney 2007, Ricciardi 2007). Humans both serve as transport vectors for invasive species (Westphal et al. 2008) and increase the amount of habitat available to them by homogenizing environments via processes such as urbanization (McKinney 2006) and agriculture (Tilman et al. 2001). In fact a close association with humans may predispose species to become invasive (Hufbauer et al. 2012) and a number of human commensals have become globally distributed (O'Connor 2013).

Invasive mosquitoes are excellent examples of taxa that have become successful worldwide invaders by virtue of their association with humans. Juliano and Lounibos (2005) found that species of mosquito that became invasive were significantly more likely to occupy human-dominated (urban and suburban) habitat, compared with non-native species that did not become invasive. From known examples, the main characteristics of invasive mosquitoes are their ability to utilize a wide range of anthropogenic containers as larval habitat, a tolerance to pollution, and the use of human blood and that of associated animals for egg production. Their choice of larval habitat also increases the likelihood of inadvertent transport by humans, for example in small containers aboard ships (Lounibos 2002). Accordingly, recent mosquito invasions have been linked to commercial importation of used tires and ornamental plants (bromeliads and bamboo) via cargo ships (Lounibos 2002, Tatem et al. 2006, Medlock et al. 2012).
Because invasive mosquitoes commonly obtain blood from humans, livestock, and wildlife, they present a significant disease risk (Lounibos 2002). Therefore, it is important to study the pathways by which they establish and spread in new areas, in order to limit expansion of existing introductions and prevent new invasions. A useful tool to illuminate pathways of dispersal and expansion in invasive species is the examination of spatial patterns of genetic variation across their exotic ranges (Miura 2007, Lawson Handley et al. 2011). Specifically, landscape genetics considers the influence of landscape features (such as roads, rivers, and mountains) on gene flow and other evolutionary processes (Manel and Holderegger 2013).

_Aedes japonicus japonicus_ Theobald (Diptera: Culicidae) is a temperate mosquito, native to northern Japan and the Korean peninsula (Tanaka et al. 1979). High-resolution spatial and temporal genetic analyses of early post-establishment populations have shed light on its recent expansion into North America and Europe (Fonseca et al. 2010, Zielke et al. 2014). In North America, it was first collected in Connecticut in 1997 (Andreadis et al. 2001) and established populations were later found in New York and New Jersey in 1998 (Peyton et al. 1999) and in Pennsylvania in 1999 (Fonseca et al. 2001). The species has subsequently spread to 31 US states, primarily in the northeast, and two Canadian provinces, as well as five countries in central Europe (Kaufman and Fonseca 2014). Fonseca and colleagues (2001, 2010) demonstrated the existence of two distinct introductions into the eastern US, defined by unique mitochondrial haplotypes and distinctive microsatellite allele frequencies: (a) the ‘PA type’ (central Pennsylvania) and (b) the ‘NY type’ (New York, New Jersey, and Connecticut). Detailed spatial and temporal analysis of specimens collected in 2002-2005 across Pennsylvania revealed
reciprocal genetic exchange between the two introductions, weakening the strong association between mtDNA haplotype and nuclear signature (Fonseca et al. 2010). Such post-establishment admixture can increase genetic diversity and generate novel combinations of genotypes within invasive species, which may lead to the emergence of traits that enhance invasiveness (Lavergne and Molofsky 2007, Keller and Taylor 2010, Ahmed et al. 2012).

*Aedes j. japonicus* commonly employs tires as larval habitat, and its arrival in the US is thought to have been associated with used tire importation (Kaufman and Fonseca 2014), but subsequent expansion may have employed other pathways. The initial detection locales in both New York and New Jersey were Standard bred horse farms, and early collections in Pennsylvania were associated with several U.S. Army bases (Fonseca et al. 2010). Eggs of *Ae. j. japonicus* may be transported in lawn ornaments, plant pots, construction supplies, and other human commercial traffic (Kaufman and Fonseca 2014). In addition to human-mediated transport of eggs/larvae along the road system, it has also been proposed that adults utilize stream corridors as natural dispersal pathways, since this species often deposits eggs in rock pools along streams, when available (Bevins 2007).

Another factor affecting the expansion of *Ae. j. japonicus* appears to be temperature. This species is primarily distributed throughout cooler latitudes in both its native (Asian) and invasive (North American and European) ranges, and its seasonal activity patterns suggest greater tolerance to cold than co-occurring native species (Kaufman and Fonseca 2014). Larvae have also been shown to survive poorly at high temperatures in laboratory experiments (Scott 2003, Egizi et al. 2014) and there is evidence that genetic patterns of this species on the island of Hawaii are strongly affected
by elevation, and its correlate, temperature (Egizi and Fonseca 2014). Temperature is known to limit the geographic distribution of another invasive mosquito, *Aedes albopictus*, but in the opposite direction: *Ae. albopictus* is sensitive to winter temperatures below -10°C and is thus limited in its northward expansion (Mogi et al. 2012).

Our goal was to test hypotheses about expansion pathways of *Ae. j. japonicus* by performing a genetic analysis of specimens collected at a fine spatial scale across the state of Virginia, which ranges from the coastal Atlantic plains to the Appalachian mountains. We were primarily interested in the following questions: (1) What are the primary dispersal pathways (landscape features/human activity) driving the expansion of this species? (2) Does temperature affect the genetic structure of populations in this cold-adapted species? (3) What can we infer about the life history and invasiveness of this species from current patterns of genetic structure?

**Methods**

We chose microsatellites as our primary population markers because of their high variability among individuals, making them useful for detecting differences in a recently introduced species, as well as their cost-effectiveness, as multiple loci can be genotyped in a single reaction. Unfortunately there are currently only seven microsatellite loci optimized for *Ae. j. japonicus* that show dependable Mendelian inheritance (Widdel et al 2005). Thus, we have also sequenced a mitochondrial locus, allowing a more comprehensive picture of genetic patterns due to the differing mutation rates and modes of inheritance of nuclear and mitochondrial DNA (Sunnucks 2000). To optimize information per individual while minimizing cost, all specimens were genotyped at the
seven microsatellite loci, but only 96 were subsampled for sequencing at the mtDNA locus.

Sample Collection and Extraction

Specimens of *Ae. j. japonicus* were collected in June-August 2011 during a distributional survey that targeted 89 independent cities and counties in Virginia with no prior collection records of this species. Larvae were collected from artificial containers (one per collection site, 80% of them tires), using 30 ml plastic pipettes or larval dippers (Clarke Dipper, Clarke, Roselle, IL) and placed on ice until they could be identified to species. A total of 1,564 *Ae. j. japonicus* larvae were identified from collections at 118 of 163 sites, comprising 79 of the 89 jurisdictions sampled. There were 10 counties where no *Ae. j. japonicus* larvae were detected despite sampling effort, all of them at low elevations along the coast (Kiser and Abadam, unpublished data).

Between 1-9 specimens from each collection site were preserved in a cryovial with 95% ethyl alcohol. When possible, different instar larvae were selected from each site to decrease the likelihood of oversampling siblings. Before DNA extraction specimens were removed from the vials allowing a few moments for any residual ethanol to evaporate, and then processed in Qiagen DNeasy blood and tissue 96 well plate kits (Qiagen, Valencia, CA). A total of 461 specimens from 100 different collection sites, comprising 74 independent jurisdictions, were used in the final analysis (Fig 3.1A).

Microsatellite Genotyping

Specimens were genotyped at seven microsatellite loci (Widdel et al. 2005) using the modified primer OJ5R3 (instead of OJ5R) to reduce null alleles, following the protocols in Fonseca et al. (2010). Microsatellite PCR products were sized on an ABI
3130XL Genetic Analyzer 16 Capillary machine (Applied Biosystems, Foster City, CA). Alleles were scored in Gene Mapper 3.5 (Applied Biosystems, Foster City, CA) using bins optimized on worldwide populations of this species (Fonseca DM unpublished data).

Mitochondrial sequencing

To subsample specimens for mitochondrial sequencing, we used a random number generator to select 32 specimens from each of 3 elevation categories (Low = below 200 m, Mid = 200-500 m, High = above 500 m). Equal representation across elevations was chosen to avoid oversampling the diversity of each, as we have previously identified elevation as a major influence on genetic diversity in this species (Egizi and Fonseca 2014). From each of these 96 specimens, we sequenced a portion of the sodium dehydrogenase subunit 4 (ND4) locus, known to be variable in this mosquito species (Fonseca et al. 2010, Zielke et al. 2014) using the same protocol as in Egizi & Fonseca (2014). PCR products were cleaned with ExoSap-IT (Affymetrix, Santa Clara, CA), cycle sequenced, and then sized on an ABI 3730XL Genetic Analyzer 96 capillary machine (Applied Biosystems, Foster City, CA). The sequences were assembled and cleaned in Sequencher 4.10.1 (GeneCodes Co., Ann Arbor, MI).

Assignment of samples to population groups

Some of the analyses required a sample size larger than 16 specimens in order to obtain the needed statistical power. Because most sites had less than 9 specimens available we grouped specimens from multiple sites to form “population groups” based on geographic proximity. Once specimens from a combination of sites surpassed the target of 16, no further specimens were added to that population group. Collection sites were excluded from these analyses if they had only 1 or 2 specimens and were isolated.
from other sites (specifically, we excluded from this subset of analyses specimens collected in Craig, Patrick, Powhatan, and Page counties and in the cities of Manassas and Falls Church). Using this method a total of 23 population groups were identified comprising 447 specimens, which were used in subsequent analyses (Table 3.1). Sites within the same population group were on average 35.23 km apart and the largest distance between any two sites within a group was 80 km. Including specimens from dissimilar sites in the same grouping is expected to increase within-population variability and obscure differences between populations, a conservative bias.

We defined the latitude and longitude coordinates of each population group as the midpoint of the GPS coordinates of all sample sites within the group, calculated using the Geographic Midpoint Calculator (http://www.geomidpoint.com/). The elevation of each population group was calculated as the average elevation of all sample sites within the group. Population groups were named by elevation category: locations collected between 0 and 200m above sea level (L = Low elevation), those collected between 200-500m (M = Mid elevation), and those collected above 500m (H = high elevation) (Fig 3.1B, Table 3.1).

*Genetic analyses*

In order to determine the ancestry of Virginia *Ae. j. japonicus* specimens (whether they have a NY or PA nuclear signature, or a mixture of both) we performed a STRUCTURE analysis (Pritchard et al. 2000), including reference specimens from the original collections in 1999 and 2000 in NY/NJ and PA (N = 25 of each), along with all 461 genotyped Virginia specimens. STRUCTURE uses a Bayesian approach to assign multi-locus genotypes into homogeneous clusters (Pritchard et al. 2000) and its
calculations are independent of any \textit{a priori} grouping. We determined the most likely number of clusters using STRUCTURE HARVESTER (Earl and vonHoldt 2012) to implement the method of Evanno et al. (2005). After STRUCTURE assigns each individual to a cluster, it gives scores for each individual estimating the proportion of its ancestry originating in each cluster (denoted by Q scores) (Pritchard et al 2000). Here, these Q scores estimate the proportion of each individual’s nuclear ancestry derived from the NY type (‘NY ancestry’) and the proportion similar to the PA type (‘PA ancestry’). We calculated the average proportion NY and PA ancestry for each collection site and population group by averaging Q scores across individuals.

Each of the original introductions to NY/NJ and to PA had a characteristic nuclear microsatellite genotype and associated set of ND4 haplotypes (‘mitotypes’) (Fonseca et al. 2010). In Virginia, we examined the correspondence between ND4 haplotype and nuclear cluster assignment (NY vs. PA ancestry) in several ways. First, we sorted each of the 96 ND4-haplotyped individuals based on whether they scored higher (>50%) for NY or PA nuclear ancestry, then used those two groups as factors in an AMOVA of mitotype frequencies in GenAlEx 6.501 (Peakall and Smouse 2006). Second, we defined NY vs. PA-associated mitotypes (H1 = NY, H9 and H12 = PA) as in Fonseca et al (2010) and performed a Chi-square test to determine whether individuals are more likely to have matching nuclear and mitochondrial designations than mixes (Table 3.2).

To visualize changes in haplotype frequency over elevation categories, we created 9 elevation categories of 100m each and plotted the proportion of NY, PA, and other haplotypes within each category. Haplotypes were also tested for an association with
elevation by defining the three elevation categories (0-200, 200-500, and 500+ m) as factors in an AMOVA on haplotype frequencies.

To compare measures of genetic diversity across population groups in relation to landscape factors, allelic richness ($A_R$) was calculated in FSTAT 1.2, standardized to the smallest population size ($N = 16$) using rarefaction (Goudet 1995) for each group. Shannon’s information index ($I$) and observed, expected, and unbiased expected heterozygosity were calculated in GenAlEx 6.501 (Peakall and Smouse 2012). Population-specific $F_{ST}$s (a measure of population differentiation) were calculated in GESTE 2.0 (Foll and Gaggiotti 2006) under default parameters. Contrary to traditional $F_{ST}$, which is calculated between populations, GESTE implements a Bayesian method that generates an $F_{ST}$ value for the deviation of each individual population from the group as a whole (Foll & Gaggiotti 2006).

**Calculation of landscape variables**

To perform Mantel tests and examine the relationship between pair-wise genetic distance and multiple landscape features, we created several distance matrices: an elevation distance matrix was calculated by subtracting the difference in elevation between pairs of population groups. A geographic distance matrix was calculated in GenAlEx 6.501 (Peakall and Smouse 2006). Matrices of distance along roads and distance along streams were calculated using the Network Analyst toolbox in ArcMap Version 10.2 (ESRI, Redlands, California). For the analysis of distance along streams, all pairs of populations that were not in the same watershed (i.e. not connected by streams) were excluded from the analysis. The genetic distance matrix used in these analyses was made from pairwise $F_{ST}$ values calculated in FSTAT 1.2 (Goudet 1995).
Temperature information for each population group was obtained from the NOAA National Climatic Data Center Mapping Tool (http://gis.ncdc.noaa.gov/map/viewer/) using the nearest weather station to the midpoint of each group. Temperature variables noted were mean annual precipitation, mean annual temperature and max summer temperature (in °C).

A list of potential used tire vendors in Virginia and their latitudinal and longitudinal coordinates were obtained from a Places search in Google Earth Version 7.1 (Google Inc., Mountain View, CA) with the terms “used tires in Virginia”. Each potential vendor was researched via the internet to confirm they sold used tires. Vendors who could not be confirmed were excluded. A total of 133 confirmed used tire vendors were identified, and the distance from each population group to the closest tire vendor was calculated using the Near tool in the Analysis Toolbox in ArcMap Version 10.2 (ESRI, Redlands, California). The distance from each population group to the nearest major highway (defined as class 1 or class 2 roads only) was also calculated using the Near tool in ArcMap Version 10.2 (ESRI, Redlands, California).

**Landscape genetic analyses**

We tested for significant values of Spearman rank order correlations between pairs of genetic variables (allelic richness, Shannon’s I, cumulative proportion PA ancestry, population-specific $F_{ST}$, observed heterozygosity and unbiased expected heterozygosity) and landscape variables (Elevation, annual precipitation, average temperature, max summer temperature, distance from used tire vendors, and distance from major highway) using the rcorr() command in the Hmisc package in R (R Core Team 2013). Spearman’s rho, a nonparametric method, was chosen due to several of the
genetic variables (namely allelic richness, proportion PA ancestry, and population-specific $F_{ST}$) failing to meet criteria of normality even after transformation. However, tests with Pearson’s $r$ returned largely the same results.

The genetic distance matrix (pairwise $F_{ST}$ values) was tested against the landscape distance matrices (straight geographic, elevation, roads, and streams) in Mantel tests in FSTAT 1.2 (Goudet 1995). Each distance matrix was first tested individually and then in a partial Mantel test controlling for straight geographic distance. Spatial auto-correlation tests were performed in GenAlEx 6.501 (Peakall and Smouse 2012) using the multilocus approach developed by Smouse and Peakall (1999). In this method an autocorrelation coefficient $r$ is calculated for the genetic distance between pairs of individuals within each geographic distance class. Then the significance of $r$ is tested both by bootstrapping (which generates a confidence interval) and a nonparametric permutation technique (which generates a $p$ value) (Smouse and Peakall 1999). This method therefore allows an examination of the spatial extent over which individuals tend to be similar. We performed spatial autocorrelation tests both across the entire dataset of individuals ($N = 461$) and at a smaller spatial scale, comparing individuals within populations with a greater proportion of NY vs. PA ancestries (see Table 3.1) roughly corresponding to the western vs. eastern parts of the state, respectively.

The program GeneticStudio (Dyer 2009) was used to create a Population Graph, a visual way of displaying genetic structure using a graph theory approach. The nodes of the graph represent populations, whose sizes are proportional to within-population variation, while the edges connect pairs of populations and represent significant covariance between them (i.e. probably reflecting gene flow) (Dyer and Nason 2004).
The sum of all edge lengths equals the among population variation component of genetic variance calculated in an AMOVA ($\sigma^2_A$) and therefore Population Graphs can be considered an extension of an AMOVA test that visualizes how the variation is apportioned rather than just testing whether it exists (Dyer and Nason 2004). Additionally, the Population Graph approach calculates genetic distance between each pair of populations, contingent on the relationships among the entire dataset, making it possibly a more informative approach than pairwise measures (such as $F_{ST}$) which consider pairs of populations in isolation (Dyer and Nason 2004). We also used the Graph component of GeneticStudio to identify compressed and extended edges in the Population Graph with a $X^2$ test. Compressed edges are genetically more different than expected, given their geographical distance, suggesting a barrier to gene flow between those two nodes. Extended edges are more similar than expected given their distance, suggesting possible long distance dispersal (Garrick et al. 2009).

**Results**

*Effect of landscape features on genetic structure*

Shannon’s $I$, a measure of between site group genetic diversity, and observed heterozygosity were both positively correlated with elevation and negatively with mean annual temperature (Table 3.3). Population-specific $F_{ST}$, a measure of each population’s relative differentiation, showed a weak positive correlation with distance from major highway, while distance from used tire vendor was not associated with any of the genetic variables (Table 3.3).

Mantel tests at the larger spatial scale, across all population groups, revealed that isolation by geographic distance explains approximately 25% of the variation in $F_{STS}$ for
the whole pairwise matrix (Table 3.4A). Both elevational separation and distance along roads show a much smaller value for $r$, once geographic distance is accounted for, although in the case of roads, it was still significant, suggesting roads do have an effect on genetic connectivity that cannot be explained by simple geographic distance. In the subset of pairwise comparisons within watersheds, streams showed a small but significant correlation with $F_{ST}$s but this ceased to be significant once geographic distance was controlled for (Table 3.4B). Spatial auto-correlation analyses across all individuals showed significant (but slight) positive autocorrelation up to distances of 160 km ($r$ declined from 0.06 to 0.01 but remained significant for all distance classes, at intervals of 20 km, up to and including 160 km). On a more local scale, comparing within eastern (PA) vs. western (NY) groupings, we detected positive spatial autocorrelation among nearby locations ($\leq 5$ km) in both ($r_{NY} = 0.07$, $r_{PA} = 0.05$, $P = 0.001$ for both).

The Population Graph shows a well-connected topology (Fig. 3.2A) suggesting there are no populations or groups of populations that are highly isolated from gene flow. The least-connected population is M10, which shares significant covariance with only 2 nodes, L2 and M4. Interestingly, these are not its closest neighbors (Fig. 3.1B). There is further evidence for widespread long-distance dispersal from the analysis shown in Fig. 3.4B, where extended edges stretch between low and high elevation populations, spanning the state. By contrast, compressed edges between pairs of populations in the mountainous west side of the state indicate possible barriers to gene flow (Fig. 3.2B).

Ancestry of Virginia specimens

Surprisingly based on the previous analysis that found extensive mixing, the eastern part of the state had a much greater proportion PA ancestry than the western part,
which had greater NY ancestry (Fig 3.3B). In fact, nuclear ancestry was significantly correlated with elevation and temperature; in particular, the PA type was associated with lower elevations (Fig 3.3A, Table 3.3) and warmer temperatures (Table 3.3).

Additionally, earlier associations between mitochondrial haplotypes and nuclear genotypes (Fonseca et al. 2010) were broadly maintained: a Chi-square examining the association between microsatellite genotype (NY vs. PA) and mitotype (specific haplotypes associated with the two introductions) was significant both when genotypes were defined as >50% ancestry ($X^2 = 8.97, p < 0.01$) (Table 3.2) and when genotypes were defined more stringently, as >75% ancestry ($X^2 = 6.59, p < 0.05$). Interestingly, introgression of the PA mitotype into NY genotypes was greater than the reverse (Table 3.2). There was also a significant difference in haplotype frequencies between NY and PA nuclear groupings from an AMOVA ($\Phi_{PT} = 0.07; P = 0.007$). Haplotype frequencies also differed between elevational groupings “low” vs. “high” ($\Phi_{PT} = 0.154, P = 0.002$) and “low” vs. “mid” ($\Phi_{PT} = 0.144, P = 0.001$), although there was no difference for “mid” vs. “high” ($\Phi_{PT} = 0.000, P = 0.363$). PA mitotypes (H9 & H12) were more common at low elevations, and the NY mitotype (H1) increased with elevation (Fig. 3.4), though this pattern broke down at elevations above 700 m due to the presence of a number of specimens with a NY nuclear genotype and H9 mitotype (Fig. 3.4).

**Discussion**

The most significant landscape factors influencing *Ae. j. japonicus* genetic connectivity in Virginia were roads. Proximity along roads explained variation in genetic distance not explained by geographic distance alone. The importance of roads to the dispersal of this species is echoed by the weak positive correlation between the genetic
differentiation of populations (measured by population-specific $F_{STS}$) and distance from major highways, suggesting that populations more distant from highways become more differentiated. Roads can be important dispersal corridors for invasive species, which either move along roads autonomously (Brown et al. 2006) or are inadvertently transported by humans (von der Lippe and Kowarik 2007), the more likely scenario for *Ae. j. japonicus*. Human-mediated transportation has been implicated as a mechanism of their dispersal within Europe, based on broad associations between new infestations and major rivers and highways (Medlock et al. 2012). In contrast, despite the possibility of streams acting as dispersal corridors for adults of this species (Bevins 2007), we found no effect of stream distance on genetic distance. And, although traffic in used tires has been invoked as a secondary dispersal mechanism for some invasive mosquitoes (Moore and Mitchell 1997), the lack of an association between genetic parameters and proximity to used tire vendors does not support such an assertion for *Ae. j. japonicus* in the eastern US. It is possible this species utilizes other container types for transportation, or alternatively, that the tires important for *Ae. j. japonicus* transport may aggregate at sites other than vendors, for example, waste piles and dumps.

Given the likelihood of *Ae. j. japonicus* being transported by humans, it is no surprise that we often found similarity between sites across the state of Virginia. While data do not yet exist for flight distances of *Ae. j. japonicus* adults, three of its congeners are only capable of dispersing up to 800 m (Watson et al. 2000, Honório et al. 2003, Russell et al. 2005, Marini et al. 2010), making diffusive dispersal by adults unlikely to explain the extended edges (greater genetic connectivity than expected) between populations at a distance of hundreds of kilometers and the positive spatial
autocorrelation at distances up to 160 km. Rather, as discussed above, it is more likely that mosquito propagules (eggs, larvae, or even adults) are being inadvertently transported large distances across the state by humans. Long distance dispersal (LDD) appears to be a common mechanism of invasive species spread (Suarez et al. 2001) and the frequency of LDD events is directly related to the rate of invasive species expansion, with a greater frequency causing a faster spread (Shigesada et al. 1995, Higgins and Richardson 1999). Human-aided LDD is also commonly invoked to explain genetic patterns in mosquitoes, when populations at great geographic distances are found to be genetically similar (Lehmann et al. 1996, Failloux et al. 1997, Huber et al. 2002). The occurrence of frequent long distance dispersal may help to explain *Ae. j. japonicus*’ rapid expansion to 30 contiguous US states in only 15 years, a rate similar to the expansion of *Aedes albopictus*, considered one of the 100 worst invaders (Benedict et al. 2007).

However, while LDD is clearly occurring, our examination of the data on a smaller scale found positive spatial autocorrelation only up to distances of 5 km, suggesting this species is also diffusing locally. The presence of compressed edges between populations along the Appalachian region indicates the mountains are acting as a barrier to gene flow, but whether they are a barrier to adult mosquito movements or to human vehicular traffic (or both) cannot be discerned. Overall, we surmise that *Ae. j. japonicus* utilizes a combination of local diffusion and human-mediated long-distance dispersal mechanisms in its expansion. The use of more than one mode of dispersal by an expanding species, termed “stratified diffusion” (Hengeveld 1989, Suarez et al. 2001), has been inferred for many invasive taxa in genetic studies (Williams et al. 2007, Darling and Folino-Rorem 2009, Walker et al. 2009, Bronnenhuber et al. 2011, Ciosi et al. 2011).
Importantly, we also found indication that temperature affects the genetic structure of *Ae. j. japonicus*. Across Virginia, there was a significantly greater proportion of PA ancestry and associated mitotypes (‘PA type’) in the east, at lower warmer elevations, vs. a greater proportion of NY ancestry and its associated mitotype (‘NY type’) at higher (colder) elevations in the west. The nuclear/mitochondrial associations from the original introductions have been largely maintained at these disparate locations in Virginia despite the fact that admixture became detectable as early as 2002 in more northern states (Fonseca et al. 2010). The results of our genetic analyses indicate two separate introductions into Virginia of populations broadly similar to the original PA and NY types, as opposed to introductions of a mixed population that later diverged. This scenario is supported by the fact that *Ae. j. japonicus* was first detected in Virginia in 2000 (Harrison et al. 2002), probably before extensive mixing between types had occurred. Our results also implicate human-mediated long distance dispersal as the mechanism by which this mosquito was introduced to Virginia, as the pattern of ancestry we observed is the opposite of what would be expected if the two types simply diffused south. The NY type was originally most common along the east coast (Fonseca et al. 2010) and the PA type most common inland, which is the reverse of their distribution in Virginia. We speculate that the two introductions arrived at different times. The 2000 detection of *Ae. j. japonicus* in Virginia was at low elevations, and early populations from low elevation areas had a strongly PA signature (Fonseca DM, unpublished). The NY introduction could have occurred later, from more mixed stock. This is supported by the observed greater introgression of PA haplotypes (specifically, H9) into NY nuclear
genotypes than the reverse, and the fact that in 2002-2003, H9 was the first PA haplotype to make an appearance in New York and New Jersey populations (Fonseca et al. 2010).

How can we reconcile the evidence of extensive LDD into and across Virginia with the significant differences we observed in genetic signature among elevations? The current distribution of genetic ancestry and diversity patterns across Virginia could be the result of historical patterns of introduction: *Ae. j. japonicus* was first detected in Occoquan, Virginia (Harrison et al. 2002), a low elevation town close to an army base (Ft. Belvoir) agreeing with the hypothesis that its spread was associated with army bases, as in PA. Indeed, all five of Virginia’s army bases are located at low elevations, where the PA type is most common. But *Ae. j. japonicus* has been in Virginia for over 10 years, and the signature of a historical accident is unlikely to be maintained in a high gene flow species without interference from some other mechanism. Therefore, although the historical introduction scenario would explain some of the observed patterns of genetic distribution, the significant association between genetic ancestry and elevation strongly implies a role for temperature-mediated selection. There is ample evidence that *Ae. j. japonicus* is a cold-adapted species whose survival is negatively impacted by high temperatures (Scott 2003, Egizi et al. 2014, Kaufman and Fonseca 2014). A study of Hawaiian populations found evidence that those at low elevations experience strong bottlenecks, resulting in lower genetic diversity and greater differentiation, compared with populations at high elevations (Egizi and Fonseca 2014). We speculate, therefore, that selection is maintaining the differing associations between mitotypes and genotypes possibly indicating differential survivorship at high temperatures. This hypothesis is further supported by the fact that mixing was prevalent above 700 m implying that
selection is strong only at low elevations, where it is warmer. Common garden experiments profiling the temperature tolerances of low and high elevation populations will be necessary to test the hypotheses of differential selection and local adaptation.

Overall, we conclude that *Ae. j. japonicus* is spreading in Virginia by a combination of frequent long distance jump dispersal mediated by human vehicular traffic and localized adult dispersal. The idea that an invasive species may rely on humans for its secondary dispersal stands in contrast to several definitions of the term “invasive species,” which presuppose a species capable of expanding by its own agency post-establishment (Ehrlich 1986, Falk-Petersen et al. 2006, Simberloff 2010). Movement by humans can influence a species’ expansion in ways that autonomous spread cannot; specifically, it can lead to large-scale admixture between locally adapted populations and potentially greater evolutionary flexibility (genetic diversity and novel genotypes) on which natural selection can act (Suarez and Tsutsui 2008, Schierenbeck and Ellstrand 2009). We observed shifts in allelic frequency with elevation in populations of *Ae. j. japonicus* that are maintained in the face of extensive gene flow, suggesting underlying selection. In this manner it appears that humans are actually driving the evolution of invasiveness in this species, an idea meriting further investigation.

**Acknowledgements**

The authors would like to acknowledge Jim Trimble and the CRSSA lab at Rutgers for use of their facilities, Dave C. Smith for advice on ArcGIS software, Dr. Peter Smouse for helpful comments on an earlier version of this manuscript, and Ann Herring for help in larval collections. This project was funded in part by USDA Hatch Grant #NJ08194 and NE-1043 Multistate funds to D. M. Fonseca.
Table 3.1 Population groupings of at least 16 samples, following Fig. 3.1B. N = sample size; $I$ = Shannon’s information index; $H_o$ = observed heterozygosity; $F_{ST}$ = population-specific FST (a measure of differentiation); Anc. = average proportion PA ancestry (NY = 1 – PA); Hwy = distance (km) from nearest major (class 1 or 2) highway; Avg. T = mean annual temperature.

<table>
<thead>
<tr>
<th>Group</th>
<th>Counties Included</th>
<th>N</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Elevation</th>
<th>$I$</th>
<th>$H_o$</th>
<th>$F_{ST}$</th>
<th>Anc.</th>
<th>Hwy</th>
<th>Avg. T</th>
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</thead>
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<td>37.56871</td>
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<td>20.79</td>
<td>1.100</td>
<td>0.539</td>
<td>0.045</td>
<td>0.733</td>
<td>6.32</td>
<td>58.0</td>
</tr>
<tr>
<td>L2</td>
<td>Sussex, Southampton, Isle of Wight</td>
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<td>36.904929</td>
<td>-76.87305</td>
<td>26.92</td>
<td>0.948</td>
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</tr>
<tr>
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<td>25</td>
<td>37.523738</td>
<td>-77.68407</td>
<td>80.67</td>
<td>0.984</td>
<td>0.533</td>
<td>0.058</td>
<td>0.675</td>
<td>1.58</td>
<td>56.5</td>
</tr>
<tr>
<td>L4</td>
<td>Brunswick, Mecklenburg, Lunenburg</td>
<td>20</td>
<td>36.814244</td>
<td>-77.97274</td>
<td>118.67</td>
<td>0.941</td>
<td>0.479</td>
<td>0.050</td>
<td>0.777</td>
<td>0.06</td>
<td>58.1</td>
</tr>
<tr>
<td>L5</td>
<td>Fluvanna, Cumberland</td>
<td>17</td>
<td>37.691306</td>
<td>-78.24706</td>
<td>130.53</td>
<td>0.960</td>
<td>0.546</td>
<td>0.053</td>
<td>0.730</td>
<td>5.28</td>
<td>58.0</td>
</tr>
<tr>
<td>L6</td>
<td>Notoway, Dinwiddie, Charlotte</td>
<td>16</td>
<td>37.105488</td>
<td>-78.04771</td>
<td>144.58</td>
<td>1.038</td>
<td>0.536</td>
<td>0.039</td>
<td>0.666</td>
<td>1.54</td>
<td>56.7</td>
</tr>
<tr>
<td>M1</td>
<td>Winchester, Frederick, Clarke, Warren, Rappahannock</td>
<td>24</td>
<td>39.022386</td>
<td>-78.13708</td>
<td>202.48</td>
<td>1.002</td>
<td>0.536</td>
<td>0.040</td>
<td>0.720</td>
<td>1.80</td>
<td>54.6</td>
</tr>
<tr>
<td>M2</td>
<td>Halifax, Pittsylvania, City of Danville</td>
<td>17</td>
<td>36.64191</td>
<td>-79.32815</td>
<td>202.59</td>
<td>1.175</td>
<td>0.617</td>
<td>0.050</td>
<td>0.549</td>
<td>3.25</td>
<td>58.5</td>
</tr>
<tr>
<td>M3</td>
<td>Buena Vista, Amherst, Buckingham</td>
<td>20</td>
<td>37.617623</td>
<td>-78.96479</td>
<td>209.86</td>
<td>0.981</td>
<td>0.511</td>
<td>0.046</td>
<td>0.809</td>
<td>2.96</td>
<td>56.4</td>
</tr>
<tr>
<td>M4</td>
<td>Appomattox, Campbell</td>
<td>16</td>
<td>37.385002</td>
<td>-78.87407</td>
<td>228.45</td>
<td>1.094</td>
<td>0.554</td>
<td>0.053</td>
<td>0.538</td>
<td>2.86</td>
<td>56.0</td>
</tr>
<tr>
<td>M5</td>
<td>Henry, City of Martinsville</td>
<td>23</td>
<td>36.736302</td>
<td>-78.98902</td>
<td>256.41</td>
<td>1.108</td>
<td>0.542</td>
<td>0.052</td>
<td>0.600</td>
<td>5.16</td>
<td>55.5</td>
</tr>
<tr>
<td>M6</td>
<td>City of Lynchburg, City of Bedford, Bedford Co, City of Salem</td>
<td>20</td>
<td>37.337952</td>
<td>-79.54375</td>
<td>276.91</td>
<td>1.112</td>
<td>0.617</td>
<td>0.051</td>
<td>0.337</td>
<td>0.18</td>
<td>56.1</td>
</tr>
<tr>
<td>M7</td>
<td>Orange, Greene, Harrisonburg, Rockingham</td>
<td>20</td>
<td>38.313187</td>
<td>-78.58897</td>
<td>301.18</td>
<td>0.936</td>
<td>0.545</td>
<td>0.082</td>
<td>0.800</td>
<td>5.80</td>
<td>55.6</td>
</tr>
<tr>
<td>M8</td>
<td>Lexington, Rockbridge, Allegany</td>
<td>16</td>
<td>37.786407</td>
<td>-79.61116</td>
<td>329.99</td>
<td>1.221</td>
<td>0.648</td>
<td>0.054</td>
<td>0.317</td>
<td>6.04</td>
<td>54.9</td>
</tr>
<tr>
<td>M9</td>
<td>Dickenson, Scott, Lee</td>
<td>20</td>
<td>36.832948</td>
<td>-82.65212</td>
<td>454.97</td>
<td>1.058</td>
<td>0.621</td>
<td>0.054</td>
<td>0.286</td>
<td>8.53</td>
<td>55.1</td>
</tr>
<tr>
<td>M10</td>
<td>Highland, Staunton, Waynesboro, Augusta</td>
<td>23</td>
<td>38.206013</td>
<td>-79.11453</td>
<td>481.66</td>
<td>1.085</td>
<td>0.559</td>
<td>0.040</td>
<td>0.650</td>
<td>0.50</td>
<td>53.6</td>
</tr>
<tr>
<td>M11</td>
<td>Covington, Bath</td>
<td>18</td>
<td>37.859177</td>
<td>-79.93192</td>
<td>483.79</td>
<td>1.257</td>
<td>0.667</td>
<td>0.022</td>
<td>0.274</td>
<td>1.42</td>
<td>54.6</td>
</tr>
<tr>
<td>H1</td>
<td>Giles, Pulaski</td>
<td>19</td>
<td>37.226357</td>
<td>-80.53314</td>
<td>572.49</td>
<td>1.046</td>
<td>0.608</td>
<td>0.125</td>
<td>0.428</td>
<td>7.62</td>
<td>51.6</td>
</tr>
<tr>
<td>H2</td>
<td>Bristol, Smyth, Washington</td>
<td>20</td>
<td>36.746573</td>
<td>-81.7987</td>
<td>612.24</td>
<td>1.115</td>
<td>0.612</td>
<td>0.048</td>
<td>0.299</td>
<td>1.93</td>
<td>53.5</td>
</tr>
<tr>
<td>H3</td>
<td>Russell, Buchanan, Tazewell</td>
<td>21</td>
<td>37.074195</td>
<td>-81.94018</td>
<td>642.01</td>
<td>1.110</td>
<td>0.571</td>
<td>0.041</td>
<td>0.423</td>
<td>7.31</td>
<td>52.4</td>
</tr>
<tr>
<td>H4</td>
<td>Galax, Carroll</td>
<td>19</td>
<td>36.691337</td>
<td>-80.93403</td>
<td>744.86</td>
<td>1.080</td>
<td>0.635</td>
<td>0.101</td>
<td>0.292</td>
<td>2.63</td>
<td>51.8</td>
</tr>
<tr>
<td>H5</td>
<td>Grayson, Wythe, Bland</td>
<td>25</td>
<td>36.844113</td>
<td>-81.13685</td>
<td>763.78</td>
<td>1.140</td>
<td>0.595</td>
<td>0.048</td>
<td>0.358</td>
<td>3.13</td>
<td>51.1</td>
</tr>
<tr>
<td>H6</td>
<td>Floyd</td>
<td>16</td>
<td>36.85804</td>
<td>-80.48116</td>
<td>837.29</td>
<td>1.122</td>
<td>0.643</td>
<td>0.039</td>
<td>0.396</td>
<td>0.03</td>
<td>51.5</td>
</tr>
</tbody>
</table>
**Table 3.2** Contingency table for Chi-square analysis on the association between nuclear and mitochondrial types. Rows correspond to nuclear ancestry (> 50%) and columns to mitochondrial haplotypes (NY = H1, PA = H9 and H12). Individuals with a non-NY or PA haplotype are excluded (N = 87).

<table>
<thead>
<tr>
<th>NY haplotype</th>
<th>PA haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY genotype</td>
<td>24</td>
</tr>
<tr>
<td>PA genotype</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 3.3 Values of Spearman’s rho (rank order coefficient) and significance values for pairwise correlations between genetic variables (columns) and landscape variables (rows). $A_R$ = allelic richness; $I$ = Shannon’s information index; Anc. = average proportion PA ancestry (NY ancestry = 1 – PA); Pop $F_{ST}$ = population-specific $F_{ST}$s (a measure of differentiation); Ho = observed heterozygosity; UHe = unbiased expected heterozygosity; Ann. Precip. = mean annual precipitation; Ann. $T^\circ$ = mean annual temperature; Max Summer $T^\circ$ = maximum summer temperature; Used Tires Dist. = distance (km) to nearest used tire vendor; Hwy Dist. = distance (km) to nearest major (Class 1 or 2) highway. Stars represent significance values: *$<0.05$, **$<0.01$, ***$<0.001$.

<table>
<thead>
<tr>
<th></th>
<th>$A_R$</th>
<th>$I$</th>
<th>Anc.</th>
<th>Pop $F_{ST}$</th>
<th>Ho</th>
<th>UHe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevation</td>
<td>0.33</td>
<td>0.54**</td>
<td>-0.72***</td>
<td>-0.02</td>
<td>0.73***</td>
<td>0.57**</td>
</tr>
<tr>
<td>Ann. Precip.</td>
<td>-0.11</td>
<td>-0.2</td>
<td>0.17</td>
<td>-0.02</td>
<td>-0.28</td>
<td>-0.13</td>
</tr>
<tr>
<td>Ann. $T^\circ$</td>
<td>-0.2</td>
<td>-0.42*</td>
<td>0.61**</td>
<td>0.06</td>
<td>-0.56**</td>
<td>-0.41</td>
</tr>
<tr>
<td>Max Summer $T^\circ$</td>
<td>-0.2</td>
<td>-0.4</td>
<td>0.52*</td>
<td>0.11</td>
<td>-0.51*</td>
<td>-0.4</td>
</tr>
<tr>
<td>Used Tires Dist.</td>
<td>-0.23</td>
<td>-0.19</td>
<td>-0.05</td>
<td>0.3</td>
<td>0.17</td>
<td>-0.03</td>
</tr>
<tr>
<td>Hwy Dist.</td>
<td>0.07</td>
<td>0.03</td>
<td>-0.07</td>
<td>0.46*</td>
<td>0.14</td>
<td>0.06</td>
</tr>
</tbody>
</table>
**Table 3.4** Mantel test results. The first variable listed is the X distance variable, and the variable following “vs.” is the Y. The presence of a second X variable following (|) indicates a partial Mantel test, where this second variable was accounted for while comparing the other two; $r =$ (partial) correlation coefficient; $P =$ p value calculated in FSTAT after 2000 permutations: (A) Results for entire pairwise matrix of population groupings. (B) Results for pairwise comparisons within single watersheds. All pairs of samples from different watersheds were excluded.

<table>
<thead>
<tr>
<th></th>
<th>$r$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Elevation vs. $F_{ST}$)</td>
<td>0.376</td>
<td>0.001</td>
</tr>
<tr>
<td>(Elevation vs. $F_{ST}$)</td>
<td>0.119</td>
<td>0.057</td>
</tr>
<tr>
<td>(Roads vs. $F_{ST}$)</td>
<td>0.507</td>
<td>0.001</td>
</tr>
<tr>
<td>(Roads vs. $F_{ST}$)</td>
<td>0.142</td>
<td>0.023</td>
</tr>
<tr>
<td>(Geographic vs. $F_{ST}$)</td>
<td>0.496</td>
<td>0.001</td>
</tr>
<tr>
<td>(Streams vs. $F_{ST}$)</td>
<td>0.417</td>
<td>0.041</td>
</tr>
<tr>
<td>(Streams vs. $F_{ST}$)</td>
<td>0.282</td>
<td>0.164</td>
</tr>
<tr>
<td>(Geographic vs. $F_{ST}$)</td>
<td>0.314</td>
<td>0.135</td>
</tr>
</tbody>
</table>
Fig. 3.1 (A) Plot of all collection sites (black diamonds) of *Ae. j. japonicus* in Virginia for specimens used in genetic analysis. (B) Plot of population group midpoints by elevation. Each group is composed of samples from neighboring counties that add up to equal a sample size of at least 16 (see Table 1 for groupings). Elevation categories are as follows: Black = High (500+ m), Gray = Mid (200-500 m), White = (<200 m).
**Fig. 3.2** (A) Network graph of population groupings of *Aedes j. japonicus* in Virginia. Nodes are colored by elevation (Black = high, Gray = mid, White = low) and node size is proportional to within-population variation. Lines connecting nodes are called edges and represent significant covariance between the two nodes, such as gene flow between populations. (B) Map of Virginia and population grouping midpoints with compressed (black dashed lines) and extended (gray solid lines) edges. Compressed edges indicate significantly less genetic similarity than expected given their geographic distance, while extended edges indicate greater similarity than expected given their distance.
**Fig. 3.3 (A)** STRUCTURE graph displaying proportion ancestry of PA type (Yellow) and NY type (Blue) in a representative sample of the original NY and PA introductions (collected in 1999 and 2000) and across modern day Virginia (collected in 2011). Virginia specimens are ordered by increasing elevation. **(B)** Plot of *Ae. j. japonicus* collection sites in Virginia, shaded by average proportion PA ancestry (calculated from STRUCTURE Q-scores) within each collection site. NY ancestry = 1- PA ancestry. Locations correspond to Fig. 3.1A.
**Fig. 3.4** Bar graph of the proportion of mitochondrial haplotypes by elevation (N = 96). X-axis is labeled with the endpoint of each bin (ex. the label ‘100’ includes observations between 0-100m, ‘200’ includes 100-200m, etc.) PA haplotypes are H9 and H12, the NY haplotype is H1, and other haplotypes (not associated with NY or PA type) are H3, H4, H6 and H11.
Conclusions and Significance

In this dissertation, I have explored mechanisms that may contribute to the invasion success of an emerging invasive mosquito: (1) habitat amelioration by co-occurring species, allowing it to exploit new geographic areas and microhabitats; (2) temperature-driven selection, leading to a more plastic species that can tolerate a broader range of environmental conditions and invade a much wider geographic area; and (3) frequent propagule movement by humans (particularly along road networks), leading to accelerated rates of expansion and population admixture.

These results will contribute to existing ecological theory on community interactions, invasion ecology, and the evolution of invasiveness. I have shown that two potentially competing species can interact in unexpected ways when mediated by a third (or more) species with a differential effect on the two competitors. This result has implications across a wide range of contexts, as no two species interact in a vacuum, and the microbial community is virtually omnipresent in nature. The results of the experiments I developed underscore the importance of species interactions and how they may change in response to modifications in the microbiota and/or environments. My results also highlight the need to re-imagine the way ecological experiments (e.g. studies of competition between pairs of mosquito species) are designed. Supporting these inferences, Bever et al. (2010) recently emphasized the importance of considering soil microbes in studies of plant community ecology. Another corollary of my findings is that invasive species with a greater tolerance to unfavorable conditions (i.e. chemicals, biotic toxins) may permit other, less tolerant species to expand their realized niche. Facilitative
interactions among invasive species have the potential to cause dramatic changes in ecosystems, accelerating the accumulation of non-natives (Simberloff 2006).

My work also has implications for understanding genetic change in invasive species, which often happens over extremely short time scales. Due to the difficulty of observing invasions early and being able to study them over a wide spatial and temporal framework, critical information is often missed. My results indicate that signatures of selection are visible in invasive populations after as little as 7-10 years. Experiments testing the temperature sensitivities of *Ae. j. japonicus* populations from low vs. high elevations in Virginia would reveal whether adaptation (phenotypic changes) to novel environmental pressures (ex. temperature) has also occurred in such a short period of time, and contribute to still emerging theory about the evolution of invasive species (Lee 2002). I have demonstrated that the predictions set forth by neutral models about the genetic signatures of a spatial expansion are inadequate to describe populations under selection. This is an important caveat to consider when reconstructing historical patterns of expansion across potentially adaptive landscapes. Finally, the speed at which invaders spread (and in turn, the speed at which they encounter novel selection pressures) may relate to their ability to exploit humans as dispersal mechanisms. Humans can transport invaders greater distances than they could reach on their own, accelerating their expansion (Shigesada et al. 1995), and increasing rates of admixture between genetically distinct populations (Schierenbeck and Ellstrand 2009). Human-mediated transportation may promote the evolution of invasiveness for some species, ensuring not only the availability of genetic diversity on which natural selection can act, but also exposing the propagules to novel selective pressures thereby driving the evolutionary process.
Understanding ecological mechanisms and evolutionary processes underlying invasion success is particularly important for invasive mosquitoes, because they often vector disease to humans, livestock, and wildlife. While *Ae. j. japonicus* is not currently considered a major disease vector in the field, it has been proven laboratory competent for several diseases of concern, including Japanese encephalitis and Rift Valley fever (Takashima and Rosen 1989, Turell et al. 2013). Its proclivity to bite humans and other mammals (Molaei et al. 2009) means it could transfer pathogens from wild and domesticated reservoir species into humans. Additionally, there is evidence from recent changes in the chikungunya virus that newly abundant mosquitoes exert a selective pressure on disease agents to adapt to them (Tsetsarkin and Weaver 2011). Therefore as *Ae. j. japonicus* continues its worldwide expansion it has the potential to become an important disease vector in the future.

Understanding how humans may be driving the evolution of invasiveness in potentially dangerous species like vector mosquitoes will hopefully lead to the development of better management strategies. One important consideration is to limit admixture by preventing additional introductions and restricting the movement of individuals between introduced populations (Suarez and Tsutsui 2008). Public education campaigns should dissuade attitudes of resignation concerning already established species. The idea that invasiveness is not an inherent characteristic but can change over time means that even if an exotic species is currently localized with minimal impact, or restricted by environmental factors (like temperature), that may not always be the case. Schemes that prioritize control efforts by evaluating the invasiveness of species should incorporate measures of their evolutionary potential (Whitney and Gabler 2008).
Appendix. Additional work on mosquito ecology

In addition to answering questions about the invasion ecology of mosquitoes in the main body of my dissertation, I have also developed tools that will aid researchers in another aspect of mosquito ecology: patterns of blood feeding (which influence their involvement in disease transmission cycles). I have developed a rapid assay to identify the host source of blood meals in the Asian tiger mosquito, an important invader and disease vector, by utilizing PCR blocking primers to prevent amplification of nuclear pseudogenes (and consequent misidentifications) (Egizi et al. 2013). In a second project, I identified both the host and sex of blood meals taken by *Culex restuans* in the early spring and found high host diversity coupled with a bias towards female hosts in female-incubating species, suggesting that *Cx. restuans* takes advantage of the nesting season to access a wider variety of hosts. This potential squandering of disease propagules on less competent hosts implies a more limited role for this species in reinitiating West Nile virus enzootic cycles than previously thought (Egizi et al. 2014). Conducting these projects introduced me to critical thinking skills and lab techniques that would later become essential to the completion of my dissertation. Citations for these two published works are listed below.


Literature Cited


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