*Am. J. Trop. Med. Hyg.*, 94(1), 2016, pp. 231–235
doi:10.4269/ajtmh.15-0351


# Evidence for an Overwintering Population of *Aedes aegypti* in Capitol Hill Neighborhood, Washington, DC

Andrew Lima, Diane D. Lovin, Paul V. Hickner, and David W. Severson*

*Disease Carrying Insects Program, Fairfax County Health Department, Fairfax, Virginia; Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana; Eck Institute for Global Health, University of Notre Dame, Notre Dame, Indiana*

*Abstract.* *Aedes aegypti* is an invasive, highly anthropophilic mosquito and a major vector for dengue and chikungunya. Population persistence in the continental United States is reportedly limited to southward of the average 10°C winter isotherm, which in the east, bisects Alabama, Mississippi, Georgia, and South Carolina. We report on summer collections and genotypic analyses of *Ae. aegypti* collected in the Capitol Hill neighborhood in Washington, DC (WDC). Analysis of a 441-bp fragment of the mitochondrial *cytochrome oxidase I* gene sequence identified the same two haplotype sequences during 2011–2014, and placed these within two discrete groups known to be derived from lineages resident in the Americas. Analysis of 10 microsatellite loci for specimens collected during 2011–2014 revealed no evidence for introgression of new alleles across years. Overall, our data support a conclusion that this represents a resident WDC population, likely maintained during winter months in a subterranean habitat that facilitates year-round survival.

*Aedes aegypti* is the primary vector of dengue, the most common arboviral disease, across most tropical and subtropical regions of the world. It is also a major vector of chikungunya, a rapidly emerging virus that is causing significant disease throughout the Caribbean islands and much of the Central and South America.[1] In the continental United States, autochthonous transmission of dengue has been observed in Florida since 2009.[2] No licensed vaccines or therapeutic drugs are available for preventing or treating either disease. *Aedes aegypti* is a highly anthropophilic invasive species that is well adapted for wide-spread colonization of urban environments, as it readily breeds in water in natural and man-made containers around and within human dwellings. Although *Ae. aegypti* has likely maintained an intermittent presence in the United States for ~375 years, its distribution has been assumed to be limited by cold temperatures to boundaries roughly southward of the average 10°C winter isotherm.[3] However, two potentially synergistic phenomena may be at play, which promote sustained northward range expansion: 1) the influence of climate changes that will allow for expansion into areas not previously colonized[4] and 2) this species readily adapts to breeding in subterranean structures that provide a suitable and protective environment from adverse climatic conditions.[5] Although *Ae. aegypti* collections have been reported from Virginia and Maryland, they typically comprised relatively small numbers and have been considered to be seasonal introductions, and restricted from overwintering by cold-season extremes,[3] as no life stage is tolerant of even short-term (e.g., overnight) temperatures below 0°C.[6] Here, we report on summer-season collections and genotypic analyses of *Ae. aegypti* larvae and adults across four contiguous years in the Capitol Hill neighborhood in Washington, DC (WDC), and conclude that this is likely an overwintering population maintained in as yet undefined subterranean habitats.

After the initial serendipitous discovery of a single adult female *Ae. aegypti* on October 22, 2011, in the Capitol Hill neighborhood, multiple subsequent surveys were performed to collect *Ae. aegypti* larvae from container habitats within ~200 m of the original collection site at the square fountain (Figure 1A). Larvae were collected from a birdbath, square fountain, trash can, potted plant saucer, and a small, grated stormwater basin in an alleyway (Figure 1B and C). Collection dates (sample sizes) were October 22 and 25, 2011 (6 and 20, respectively); September 26, 2012 (17); July 13, August 25, October 19, and November 22, 2013 (6, 1, 8, and 1, respectively); and August 18 and September 14, 2014 (49 and 7, respectively). A total of 115 *Ae. aegypti* larvae were identified using standard taxonomic keys.[7] Adults were occasionally reared from larvae, killed by freezing, and dried by placing them in open glass vials. Larvae were killed with hot water then stored in 2.5-mL Eppendorf tubes containing 70% ethyl alcohol. Samples were shipped to University of Notre Dame for genetic analysis.

Ethanol-preserved larval and dried adult samples were subjected to DNA extraction as previously described,[8] and DNA was suspended in final volumes of 200 μL for larvae and 1,000 μL for adults containing 0.02 M NaOH and 0.036 M Tris-HCl, pH 8.0. A 710-bp amplicon from the mitochondrial *cytochrome oxidase I* (*mtCOI*) gene was obtained using LCO1490 and HCO2199 primers in 25 μL PCR reactions containing 1 μL of genomic DNA and was subjected to thermocycling conditions as previously reported.[9] Amplicons were purified (Qiagen QIAquick PCR Purification Kit) and direct sequenced with the same primers using BigDye on a 3730*xl* DNA Analyzer (Applied Biosystems). Multiplexes of primers for 10 microsatellite markers with fluorochrome-labeled forward primers (Integrated DNA Technologies) were prepared in 25 μL PCR reactions, amplified, and subjected to fragment analysis as previously described.[8]

Fifty-nine *mtCOI* sequences were obtained and aligned in Clustal Omega[10] with 20 *Ae. aegypti mtCOI* sequences from various geographic regions of the world downloaded from VectorBase.[11] All sequences were trimmed to a 441-bp consensus and used to construct a neighbor-joining phylogenetic tree in MEGA6,[12] using a *mtCOI* sequence from *Aedes albopictus* (GenBank accession no. KC690960) as an outgroup. For microsatellite analysis, samples were divided by year of collection (YOC) and tested for Hardy–Weinberg equilibrium (HWE) using Arlequin v3.5.[13] The Bayesian clustering method in the STRUCTURE program v2.3.4[14] was used to assign individuals to genetic clusters. We implemented

*Address correspondence to David W. Severson, Department of Biological Sciences, University of Notre Dame, 107C Galvin Life Sciences, Notre Dame, IN 46556. E-mail: severson.1@nd.edu

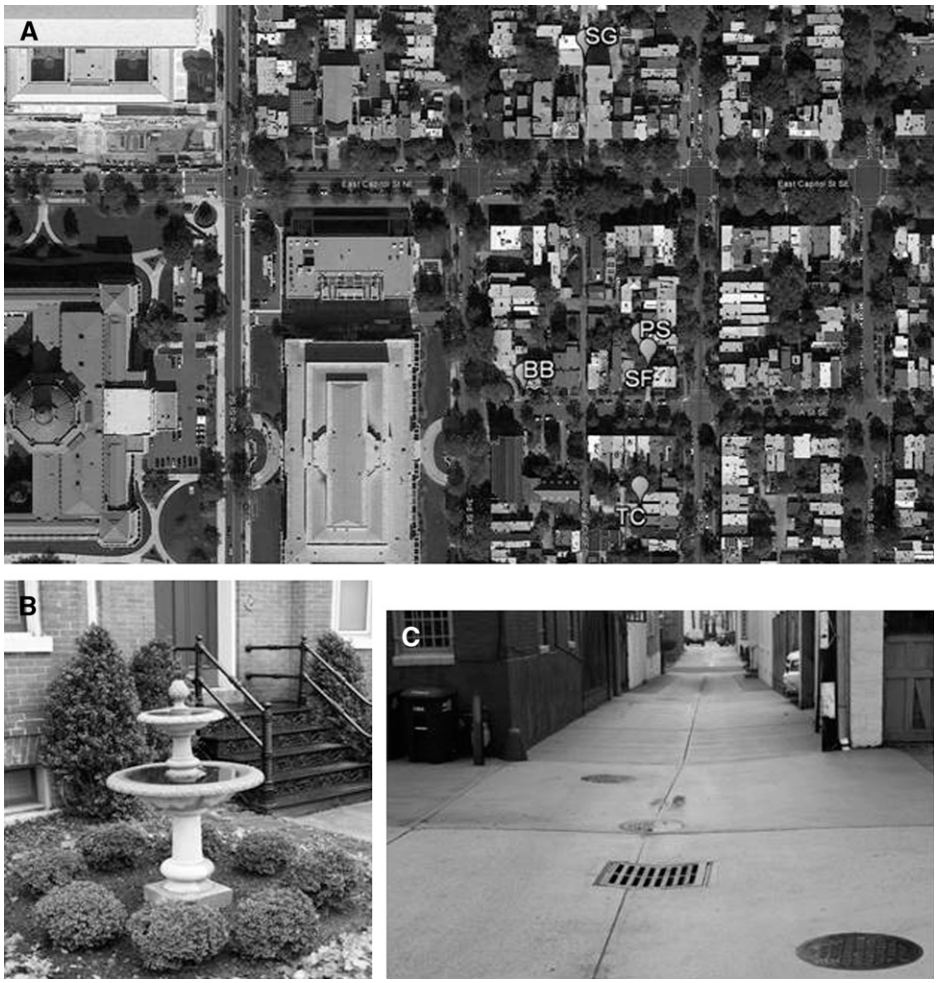


FIGURE 1. (**A**) *Aedes aegypti* collection sites in Capitol Hill neighborhood of Washington, DC, 2011–2014. BB = birdbath; SG = storm sewer grate; SF = square fountain; TC = trash can ; PS = potted plant saucers. Library of Congress building is on lower left. The figure was generated using Google Earth Pro. (**B**) BB breeding site, where larvae were detected during all four collection years. (**C**) SG breeding site, where water stands 0.3–0.6 m below street level. This figure appears in color at www.ajtmh.org.

the Evanno method[15] to determine the most likely number of clusters (K) after five independent runs of STRUCTURE for K = 1 to K = 5 on individuals from four YOC, using 100,000 burn-in steps followed by 500,000 iterations.

We identified two *mtCOI* haplotypes from a total of 59 sequences obtained from *Ae. aegypti* individuals collected during 2011–2014. The two haplotypes were shared across all four YOC, and were often identified in larvae collected from the same breeding container such as the birdbath (Figure 1B). Our sequence data were submitted to GenBank under accession numbers KM362421 and KM362422. The phylogenetic tree based on a combined analysis of the WDC sequences with 20 *mtCOI* haplotype sequences from various geographic regions placed the WDC haplotypes within two distinct phylogenetic groups (Figure 2A), reflective of the two African origin ancestral clades associated with extant global *Ae. aegypti* populations.[16] The most common WDC haplotype A showed close relationships with *Ae. aegypti* populations from South America and Asia, whereas WDC haplotype B showed close relationships with *Ae. aegypti* populations from the Caribbean, South America, and sub-Saharan Africa. This finding is completely consistent with results obtained from multiple studies on *Ae. aegypti* populations throughout the Caribbean and Central and South America and with data from multiple mitochondrial genes.[17–19] This and the fact that the two haplotype sequences are identical to the haplotype sequences reported for *Ae. aegypti* populations in Brazil and Guyana support the conclusion that the WDC *Ae. aegypti* population is derived from lineages resident in the Americas, and were likely transported into WDC via human-mediated travel. These data also support the expected outcome of a persistent low genetic diversity founder population; for example, in contrast, analyses of *Ae. aegypti mtND4* and *mtND5* haplotypes across southern Florida in 2006 revealed 68 different haplotypes among 362 individuals examined.[19]

We determined genotypes at 10 microsatellite loci for 70 individuals from the WDC population collected in 2011 ($N$ = 22), 2012 ($N$ = 12), 2013 ($N$ = 8), and 2014 ($N$ = 28). These loci fit HWE expectations with only two exceptions across all years, with the total number of alleles observed per locus across all years ranging from two to 10 (Figure 3). Examination of individual allele presence and frequency across years clearly reflect no evidence of new allele introgression

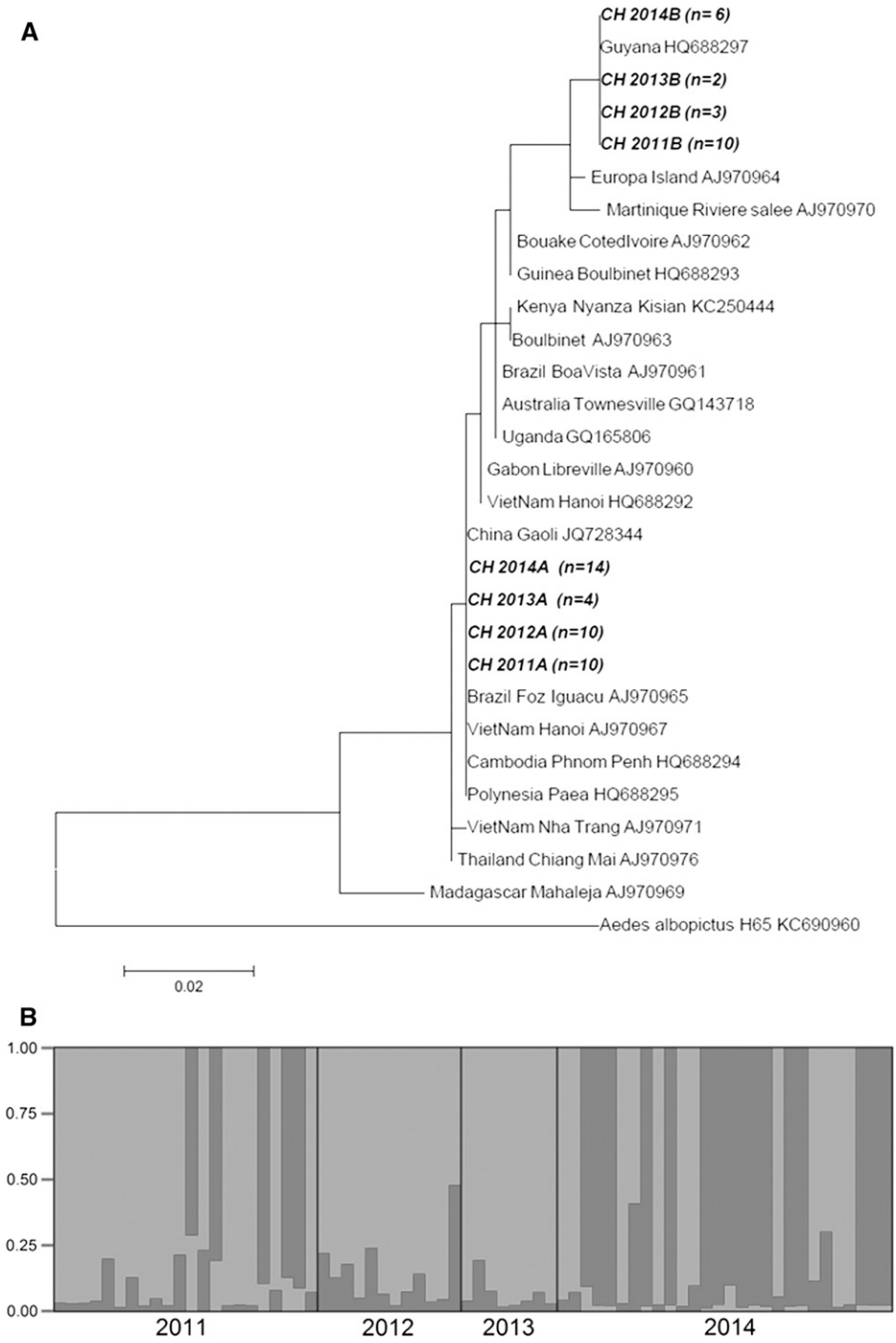


FIGURE 2. (**A**) Phylogenetic tree constructed with a consensus 441 bp of the mitochondrial *cytochrome oxidase I* gene sequence. The analysis included sequences from 59 individuals collected in Washington, DC, during 2011–2014 and 20 sequences from various geographic regions obtained from VectorBase[11] and using an *Aedes albopictus* sequence as an outgroup. (**B**) Cluster analysis of 10 microsatellite loci for 70 individuals collected in Washington during 2011–2014 using STRUCTURE.[14] K = 2, where each vertical bar represents a single individual and each color reflects probability of assignment to a cluster. This figure appears in color at www.ajtmh.org.

across all 10 loci, further supporting our conclusion that this represents an overwintering population in WDC. Bayesian cluster analysis using STRUCTURE identified the likely number of clusters as K = 2, but failed to detect any evidence for clustering across YOC as the WDC annual populations represented similar mixtures of the identified genetic clusters (Figure 2B).

Taken together, our *mtCOI* haplotype and microsatellite marker data confirm consistent genetic identities among *Ae. aegypti* WDC populations across all YOC, with no evidence of introgression by novel genotypes. The most parsimonious conclusion is that this represents a small continuous resident WDC population that reflects founder effects since the original introduction during or prior to our initial sampling

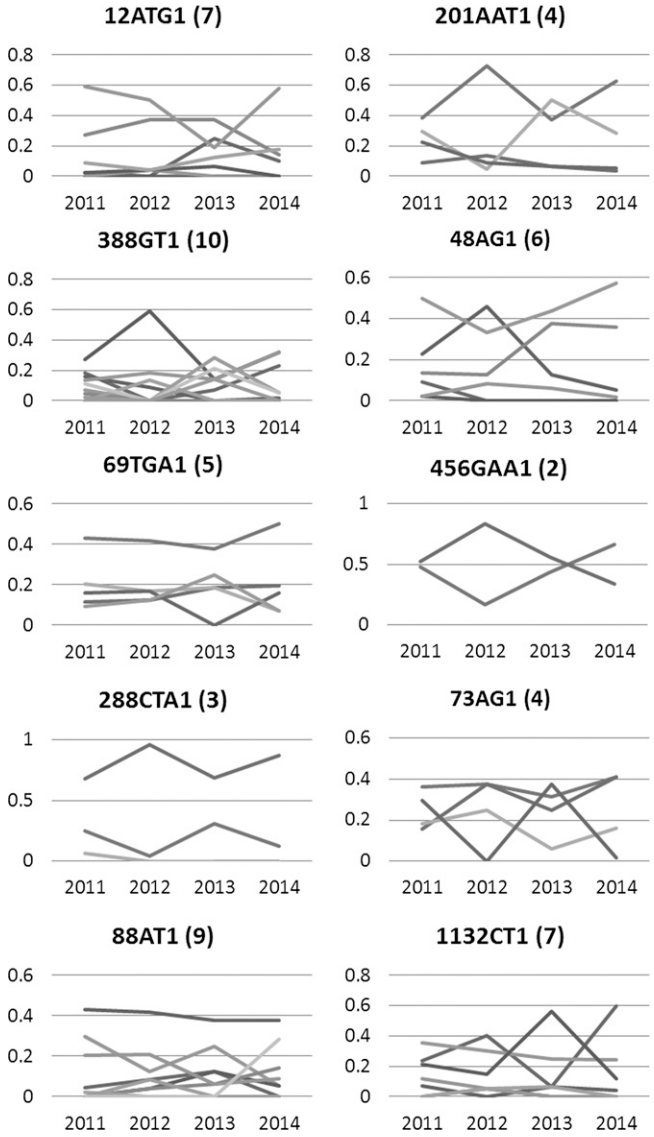


FIGURE 3. Allele frequencies observed for microsatellite loci among individuals collected in Washington, DC, during 2011–2014. Numbers in brackets indicate number of alleles per locus. This figure appears in color at www.ajtmh.org.

in 2011. This raises interesting questions about how the population has persisted despite the obvious extreme winter seasonal conditions, with an average January isotherm for WDC of only 2.2°C.[20] A historical review of *A. aegypti* presence across the continental United States does include documented collections in the northern Virginia/Maryland/WDC area, but concludes that this represents the extreme northern edge and that overwintering populations were unlikely.[3] January isotherms between collection years had considerable variation, with two consecutive mild years followed by extraordinary cold (2012: 4.9°C, 2013: 4.6°C, and 2014: 0.17°C). The most likely scenario is that an *Ae. aegypti* population has colonized a subterranean habitat with suitable climate, periodic standing water, and potentially access to bloodmeal sources for year-round breeding and survival. However, as *Ae. aegypti* eggs and larvae can survive much lower temperatures than adults,[6] a subterranean habitat may be compatible for winter survival as diapausing eggs. We speculate that presently unknown point(s) of access from the subterranean site(s) to the surface must exist within the immediate area around the collection sites (Figure 1B and C) and that small numbers of adults are finding the route to the surface and engaging in limited breeding each summer.

Received May 12, 2015. Accepted for publication September 29, 2015.

Published online November 2, 2015.

Disclaimer: The findings and conclusions presented are solely those of the authors and do not necessarily reflect the views of their respective institutions.

Authors' addresses: Andrew Lima, Disease Carrying Insects Program, Fairfax County Health Department, Fairfax, VA, E-mail: andrew.lima@fairfaxcounty.gov. Diane D. Lovin, Paul V. Hickner, and David W. Severson, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, and Eck Institute for Global Health, University of Notre Dame, Notre Dame, IN, E-mails: dlovin@nd.edu, phickner@nd.edu, and severson.1@nd.edu.

## REFERENCES

1. Morens DM, Fauci AS, 2014. Chikungunya at the door—déjà vu all over again? *N Engl J Med 371:* 885–887.
2. Anez G, Rios M, 2013. Dengue in the United States of America: a worsening scenario? *BioMed Res Int 2013:* 678645.
3. Eisen L, Moore CG, 2013. *Aedes* (*Stegomyia*) *aegypti* in the continental United States: a vector at the cool margin of its geographic range. *J Med Entomol 50:* 467–478.
4. Campbell LP, Luther C, Moo-Llanes D, Ramsey JM, Danis-Lozano R, Peterson AT, 2015. Climate change influences on global distributions of dengue and chikungunya virus vectors. *Phil Trans R Soc B 370:* pii: 20140135.
5. Russell BM, Foley PN, Kay BH, 1996. The importance of surface versus subterranean breeding sites for mosquitoes during winter in north Queensland. *Arbovirus Res Aust 7:* 240–242.
6. Davis NC, 1932. The effects of heat and cold upon *Aedes* (*Stegomyia*) *aegypti. Am J Hyg 16:* 177–191.
7. Darsie RF, Ward RA, 2005. *Identification and Geographical Distribution of the Mosquitoes of North America, North of Mexico*. Gainesville, FL: University of Florida Press.
8. Lovin DD, Washington KO, deBruyn B, Hemme RR, Mori A, Epstein SR, Harker BW, Streit TG, Severson DW, 2009. Genome-based polymorphic microsatellite development and validation in the mosquito *Aedes aegypti* and application to population genetics in Haiti. *BMC Genomics 10:* 590.
9. Hemme RR, Thomas CL, Chadee DD, Severson DW, 2010. Influence of urban landscapes on population dynamics in a short-distance migrant mosquito: evidence for the dengue vector *Aedes aegypti*. *PLoS Negl Trop Dis 4:* e634.
10. EBI, 2014. *Clustal Omega*. Available at: http://www.ebi.ac.uk.
11. VectorBase, 2014. *Aedes aegypti*. Available at: http://www.vectorbase.org.
12. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol 30:* 2725–2729.
13. Excoffier L, Laval G, Schneider S, 2005. Arlequin ver. 3.5: an integrated software package for population genetics data analysis. *Evol Bioinform Online 1:* 47–50.
14. Pritchard JK, Stephens M, Donnelly P, 2013. Inference of population structure using multilocus genotype data. *Genetics 155:* 945–959.
15. Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol 14:* 2611–2620.
16. Moore M, Sylla M, Goss L, Burugu MW, Sang R, Kamau LW, Kenya EU, Bosio C, de Lourdes Munoz M, Sharakova M, Black WC, 2013. Dual African origins of global *Aedes aegypti*

s.l. populations revealed by mitochondrial DNA. *PLoS Negl Trop Dis 7:* e2175.

17. Mousson L, Dauga C, Garrigues T, Schaffner F, Vazeille M, Failloux AB, 2005. Phylogeography of *Aedes* (*Stegomyia*) *aegypti* (L.) and *Aedes* (*Stegomyia*) *albopictus* (Skuse) (Diptera: Culicidae) based on mitochondrial DNA variations. *Genet Res 86:* 1–11.
18. Bracco JE, Capurro ML, Lourenco-de-Oliveira R, Sallum MAM, 2007. Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence for multiple introductions. *Mem Inst Oswaldo Cruz 102:* 573–580.
19. Damal K, Murrell EG, Juliano SA, Conn JE, Loew SS, 2013. Phylogeography of *Aedes aegypti* (yellow fever mosquito) in south Florida: mtDNA evidence for human-aided dispersal. *Am J Trop Med Hyg 89:* 482–488.
20. NOAA, 2015. *Washington D.C. Average Temperatures*. Available at: http://www.erh.noaa.gov/lwx/climate/dca/dcatemps.txt. Accessed March 26, 2015.