Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

______________________________ _______________

Karen Wu Date

Factors that affect *Culex* mosquito feeding choice in Atlanta, Georgia

By

Karen Wu
Degree to be awarded: Master of Science in Public Health

Global Epidemiology

____ [Chair's signature]
Gonzalo Vazquez-Prokopec
Committee Chair

____ [Member's signature]
Uriel Kitron
Committee Co-Chair

____ [Member's signature]
Paula Marcet
Committee Member

Factors that affect *Culex* mosquito feeding choice in Atlanta, Georgia

By

Karen Wu

Bachelor of Science
Emory University
2010

Thesis Committee Chairs:
Gonzalo Vazquez-Prokopec M.Sc. Ph.D.
Uriel Kitron Ph.D. M.P.H.

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Public Health
in Global Epidemiology
2013

Abstract

Factors that affect *Culex* mosquito feeding choice in Atlanta, Georgia
By Karen Wu

Background

Since its introduction in 1999, West Nile Virus (WNV), a mosquito-borne zoonosis, has spread throughout the United States. Enzootic transmission occurs between female mosquitoes of the genus *Culex*, and various passerine birds. Human, who are dead-end hosts, can become infected following the bite of an infected mosquito. Therefore, any factors that may affect mosquito host choice are likely to have an impact on the probability of t WNV transmission to humans. A feeding shift from avian to mammalian species may contribute to the WNV transmission patterns in the eastern U.S. In addition, mosquito host choice is one of the key parameters of mathematical models that predict pathogen transmission.

Methods

A total of 86 blood fed mosquitoes were processed from Tanyard Creek and Peavine Creek in Atlanta, Georgia over two years, 2010 -2011. The mosquitoes were analyzed for blood meal sources using hemi-nested PCR protocols targeted at the 16S ribosomal gene. The samples were then sequenced in order to identify the host to the species level. Fisher's exact tests were performed on dichotomized variables such as creek, year, and season. In addition, a logistic regression was performed to identify any significant additional predictors that could yield information on mosquito feeding preference.

Results

The Fisher's exact test suggested that the dichotomous variable creek was significantly associated with the number of blood meals that were avian or human in origin ($p=0.0163$, $p=0.0231$) while year is significantly associated with the number of human blood meals ($p=0.0002$). Results from a stepwise logistic regression suggested that creek was the only significant predictor associated with mosquito feeding preference between birds and humans and year was the only predictor with significantly associated with feeding preference between human versus nonhuman blood meal sources ($p<0.001$). Month and season were not associated with any blood meal preference.

Conclusion

The significant association of creek and year with feeding preference is in agreement with the notion that *Culex spp.* are a opportunistic feeders that can shift between blood meal sources, rather than between hosts from one season to another. This opportunistic nature is in agreement with the hypothesized role of *Culex spp.* as vectors for enzootic transmission and as bridge vectors to humans.

Factors that affect *Culex* mosquito feeding choice in Atlanta, Georgia

By

Karen Wu

Bachelor of Science
Emory University
2010

Thesis Committee Chairs:
Gonzalo Vazquez-Prokopec M.Sc. Ph.D.
Uriel Kitron M.P.H. Ph.D.

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Public Health
in Global Epidemiology
2013

Acknowledgements

I would like to thank Drs. Gonzalo Vazquez-Prokopec and Uriel Kitron for their continuous assistance, support and guidance throughout the duration of this project. In addition, the collaboration within the Kitron/Vazquez-Prokopec lab, especially with Rebecca Levine and JR McMillan, gave invaluable support and insight into this project.

This project would not have been possible without the support of Dr. Paula Marcet and the Centers for Disease Control and Prevention. I sincerely appreciate the guidance, the advice, and your continuous push to make this project great.

Lastly, I would like to thank my friends and family for their endless love and support throughout this whole process.

Table of Contents

Introduction 1
Methods 7
Field sampling 7
Blood meal identification 8
Data processing and analysis 13
Results 14
Mosquito collections, species, Sella scores 14
Blood meal analysis 15
Analytic statistics 16
Discussion 17
Future Directions 23
Tables 33
Figures and Graphs 40
Appendix A: Laboratory protocols 47

Introduction

Zoonotic diseases have become increasingly important in public health in recent years due to their increasing incidence and their continuous emergence. An emerging disease is defined as a disease that is caused by either a novel human pathogen, or a previously existing pathogen that has spread into new areas, or into previously eliminated areas, and therefore is introduced into susceptible populations. About 60.3% of emerging infectious disease outbreaks of pathogens newly found in humans are zoonotic in nature (1). The increase of spillover of pathogens from animals to humans is expected to continue, as zoonotic diseases are difficult to prevent due to their complex transmission cycles and multifaceted linkages between humans and the environment. Many factors have been found to trigger differences in human disease incidence, including shifts in the range of the disease, change in human population density, fluctuations in the prevalence of infection in host or vector, changes in pathogen load, or variability in the climate or weather (2, 3). Emerging infectious diseases in recent years have been commonly associated with human mediated environmental changes. The urbanization process has been found to facilitate the spread of zoonotic diseases primarily by altering the human-wildlife interface upon which transmission occurs. The encroachment of people upon lands previously inhabited by wildlife increases contact between animals and humans and therefore increases the likelihood of wildlife disease spillover into the human population (4). For example, the emergence of Nipah virus among humans in Malaysia is hypothesized to be related to the habitat destruction of its reservoir host, the fruit bat (5, 6). The destruction of habitat led to wildlife encroachment into farmlands that bred

swine, which contracted Nipah virus. Swine, in turn, transmitted the virus to humans, and caused 265 cases, 105 deaths, and the culling of one million pigs from late September 1998 to June 1999 (7). In addition, the population density within areas that encourage interaction between wildlife and humans is steadily increasing due to the natural progression of human movement (8).

West Nile Virus (WNV) is a mosquito-borne, zoonotic disease that was first detected in the United States in New York City in 1999 (9). By 2005, WNV had spread to all 48 contiguous U.S. states (10). WNV causes severe neuroinvasive disease in less than one percent of people that contract the virus, and of those neuroinvasive cases, 11-12% are fatal (9, 11, 12). Approximately 80% of people who contract the virus are asymptomatic while the other 20% develop West Nile fever, which includes symptoms such as fever, headache, fatigue, body aches, and a macular rash. The asymptomatic and mild symptoms lead to underreporting of all persons infected with WNV, and therefore, most of the surveillance systems emphasize the detection of neuroinvasive WNV (11). There were 2,734 cases of neuroinvasive WNV reported within the United States in 2012- a significant increase from the 486 cases in 2011, which suggests that West Nile Virus is well established and will be a continuing, increasingly important, public health issue (10).

WNV is maintained in an ecosystem through an enzootic cycle primarily consisting of avian species, with spillover to humans as dead-end hosts. Humans are infected when a mosquito feeds on an infected bird, becomes infected, and then subsequently feeds on a person. However, humans are considered a dead-end host and will not contribute to further vector-borne transmission. The process through which a

human gets infected involves a series of contacts between mosquito and avian species, and mosquitoes and humans. The community composition of the vector and host has been identified as a factor that significantly affects levels of zoonotic pathogen transmission supported by hypotheses such as the dilution effect, coined by Ostfeld and Keesing (13, 14). The dilution effect suggests that an increase in host community diversity will decrease the prevalence of infected vectors by reducing the probability of a vector feeding on competent hosts. For WNV the competency of avian hosts are determined by susceptibility to the virus, infectiousness, as well as duration of the viremia (15). In support of the dilution effect, increased avian diversity has been shown to be correlated with a decrease in the prevalence of WNV (16-18). However, the applicability of the dilution effect is based upon several key assumptions, including the following: the presence of a generalist vector, the primary acquisition of the virus must be by host feeding, there must be a variation in host competence, and there must be a correlation between host competence and host numbers in the community (19). These unmeasured relationships and assumptions may have an unknown, yet significant impact on pathogen transmission (20).

In addition, predictive formulas that allow for the calculation of cases, such as vectorial capacity, employ several assumptions that involve preferential feeding. Vectorial capacity describes the daily rate at which future inoculations occur from an infective case, and therefore is an extremely important calculation in describing pathogen transmission (21). The vectorial capacity formula assumes that the interactions between vector and hosts are uniform- that is, the vector has no host preference (21). The importance of further knowledge of blood feeding may significantly affect how models

are made in the future, improving both general knowledge and accuracy of predictive models.

To assess the vector for generalist versus preferential feeding properties, the likely vector for WNV must be identified. The vector must be competent, which is determined by the infection rate, dissemination rate, and the ability to transmit the virus to a host (22), and the vector must feed on both avian and mammalian species. *Culex spp.* mosquitoes have been identified as a genus that is both competent and likely to transmit WNV by their feeding behaviors (23-25). *Culex pipiens* has been identified as a primarily ornithophilic feeder, while *Culex pipiens quinquefasiatus* has been found to be more opportunistic in nature in the United States (26-31). The presence of a generalist vector has been in question in the West Nile Virus system. In Africa, *Culex quinquefasiatus* has been identified to be highly anthropophilic (32). However, in the U.S., *Culex pipiens* and *Culex pipiens quinquefasciatus* have been found to hybridize readily (33-35). Detailed studies have shown a disparity in *Culex pipiens complex* feeding preference based upon location (28). Therefore, assumptions on the feeding preference of *Culex* mosquitoes should be made on a location basis. Mosquito species that commonly feed on both avian and mammalian hosts need to be behaviorally characterized in detail, as they may be the vector for pathogen spillover of WNV into the human population. Various studies have drawn associations between feeding preference and possible predictors, but not with the mosquito population in Atlanta, Georgia.

Previous studies have yielded information suggesting shifts in feeding preferences of *Culex* mosquitoes. A study on *Culex pipiens* blood meals in the mid-Atlantic (Maryland and Washington D.C.) suggests that there is a shift from American robins to

humans in late summer (36). Other *Culex spp.* have been found to shift in feeding choice in early summer from avian to mammals, and shift back to avians in the fall season (37). A shift from avian species (such as the American robin) to mammalian species would facilitate the transmission of WNV from reservoir to humans. The epidemic curve for WNV reflects an increase in cases at the end of June, which is similar to previously identified *Culex spp.* shifts in feeding patterns. However, no shift was detected in studies performed in other areas such as California and Louisiana (29, 38), suggesting that it is difficult to justify a shift that can be generalized across urban areas within the United States, due to the difference in temperatures, land cover, and location in relation to an avian species' migration pattern. Optimally, data on mosquito feeding must be collected and analyzed on a species and location basis.

In the state of Georgia, the incidence of neuroinvasive WNV is considered to be low, given its specific ecological characteristics. The urbanization of areas affects community composition and can increase both human-wildlife contact (39), usually decreasing the biodiversity in a system. According to the dilution effect, a decrease in biodiversity would be associated with an increase in WNV cases (17, 18). In addition, WNV has been associated with a higher vegetation index and forest cover (40, 41), and Georgia has been found to have the highest percentage, 55.3%, of tree cover in urban areas in the U.S. (42, 43). Therefore, we would hypothesize that Georgia would have a relatively high incidence of WNV. However, this is contrary to what is observed (Figure 1). The varying incidence of WNV in various U.S. states is hypothesized to be possibly associated to the biodiversity wildlife communities present in the area and, as discussed previously, a consequence of the dilution effect.

The interaction between hosts and vectors can be inferred and characterized using blood meal identification techniques, such precipitin tests, ELISA, and PCR (44-46). The usage of a PCR-based method allows for the detection of the blood meal to the species level (47). The 16S ribosomal RNA gene has been commonly used to identify species of bacteria, bed bugs, amphibians, as well as the hosts of blood fed sand flies (48-52). 16S was shown to be a valid gene for identifying both mammalian and avian mosquito blood meals at the University of Georgia, Southeastern Cooperative Wildlife Disease Study and at the University of Tennessee, Knoxville. The 16S gene is highly conserved as it codes for a ribosome, but still had base-pair mismatches that were identified through orthologs from several families (52). Therefore, this gene was confirmed to be highly conserved among organisms, which allows the design of a unique molecular marker for a wide variety of organisms, but yet is different enough to be useful for phylogenetic classifications, as species will present with different sequences.

The public health importance of this project is based upon the ability to improve public health control measures in an urban setting by characterizing the feeding choice of the mosquitoes of interest. We hypothesize that *Culex pipiens quinquefasciatus* displays an opportunistic feeding behavior based upon the host community composition, and we expect to see a shift from avian species to mammalian species in mid summer, which would explain the phenomena depicted in the seasonal transmission of WNV in Atlanta. Lastly, we aim to detect additional predictors that may aid in describing significant differences in host preference, such as year and location.

Methods

Field sampling

Field collections took place at multiple sites along two creeks located in Atlanta, Georgia (Figure 2). The creeks were approximately 6.8 km apart and the sampling sites were distanced approximately 25 to 150 meters apart (Figure 2). Collections were made on a weekly basis from May to October in June-November 2010 and May-September 2011. Mosquitoes were collected using a gravid trap set overnight with a hay and dog food infusion, and aspirated from surrounding vegetation using a Prokopack mosquito aspirator for approximately ten minutes upon gravid trap pick-up (53, 54).

Collected mosquitoes were identified based on morphological characters using the guide, *Identification and geographical distribution of the mosquitoes of North America, north of Mexico* (55). Species that were captured and classified as *Culex pipiens* are assumed to be *Culex pipiens quinquefasciatus (56).* Mosquitoes were also classified by Sella score (Figure 4). Sella score, which ranges from one to seven (1 = unfed, 2-6 = fresh to partially digested blood meal, 7 = gravid), is frequently used to determine the freshness of the blood meal based upon visual characteristics (57, 58) (Figure 4). The blood fed mosquitoes were placed in individual 1.5 mL tubes (Eppendorf, Hauppauge, New York) with one mL of Minimum Essential Medium Eagle with Antibiotic Antimycotic Solution (Sigma Aldrich) and two CopperHead BBs (Crosman Corporation, Bloomfield, NY). The samples were stored at -80°C until testing for WNV. Prior to testing, the mosquitoes were rapidly homogenized using the two copper pellets and a Biospec Mini Bead Beater-8 at high speed. The subsequent solution was used to inoculate

African green monkey kidney cells to test for West Nile Virus (WNV). All blood fed mosquitoes used for blood meal analysis tested negative for WNV.

Blood meal identification

All blood fed mosquito samples were extracted using a QiaAmp Viral RNA Mini Kit (Qiagen Sciences). The extracted samples were stored at -20°C until use. Mosquitoes with a Sella score of six were excluded for further blood meal processing due to the likelihood of digested and therefore degraded and unidentifiable DNA. In total, 86 *Culex* mosquitoes were processed and all mosquitoes missing all taxonomic information, Sella scores and capture site were removed from analysis.

DNA controls for PCR quality were extracted from a *Dumetella carolinensis* (Gray Catbird) sample using a QIAamp DNA Mini and Blood Kit (Qiagen Sciences), while blood clots from *Scicurus carolinensis* (Eastern Gray Squirrel), were extracted using a DNeasy Blood and Tissue Kit (Qiagen Sciences). All the extractions were performed under manufacturer recommendations.

A hemi-nested PCR protocol was used to determine the host on which the mosquito fed upon. A hemi-nested protocol involves two separate PCR reactions in order to increase the sensitivity, which is especially useful in cases with low amounts of template DNA. The first reaction amplifies a region larger than the target, and the product is then used as a template for the second reaction. Since the reverse primer stayed constant, and only the forward primer was nested within the product of the first reaction, the protocol is determined to be hemi-nested.

The primers were designed by Killmaster et al. (52)(Table 1). Two independent hemi-nested reactions were performed with each sample, one targeting a mammalian

DNA product, and the other targeting an avian product. The final sequence was expected to be approximately 550 base pairs for both reactions. Original PCR conditions were optimized to increase sensitivity and specificity by changing the total volume of the reaction, the concentration of magnesium chloride, number of cycles for the second reaction, and the final elongation time. The final conditions at which all the samples were processed can be found in Appendix A. The master mix and PCR samples were prepared under a biosafety hood and surfaces were frequently decontaminated with bleach and ethanol in order to discourage any possible contamination. Any objects entering the hood were wiped down with bleach as well. All reactions included a negative control (without any DNA) to evaluate for unspecific amplification and contamination of reagents, and a positive control (with mammalian and avian DNA) for PCR conditions and reagents.

Avian and mammalian DNA controls were isolated from blood clots. A sample collected from a Gray Catbird was used as a positive avian control, and a human sample (provided by the Centers for Disease Control and Prevention, Parasitic Diseases Branch) or an Eastern Gray Squirrel sample was used for the mammalian positive control.

A 1.5% agarose gel containing ethidium bromide was used to determine PCR success by direct observation of the PCR product using a UV light. If the PCR was not successful or yielded smeared bands, the hemi-nested reaction was repeated. If the second reaction failed to successfully amplify the DNA target, the reaction was considered negative. If the second reaction failed to yield a clear band, the sample was either considered negative or degraded. Results were determined considering the amplification obtained in each of the reactions for each sample, which were classified as avian, mammalian, or mixed (both avian and mammalian) blood meal sources.

When the sample amplified successfully, the PCR product was purified using a Millipore MultiScreen plate following the manufacturers recommendations (Appendix B). The DNA was then re-suspended in TE buffer and stored at -20°C in a 48-well PCR plate (USA Scientific). The amount of TE used per well was based upon the amount of DNA estimated from the brightness of the band observed in the gel.

Prior to sequencing, a cycle sequence reaction (Applied Biosystems) was performed in order to attach a fluorescent dideoxynucleotide to random points in the amplified sequence. The attachment of each dideoxynucleotide terminates the replication at that point, creating a plethora of fragments terminated at random points within the DNA. This process is necessary in order to sequence the fragment, by making the PCR product detectable by an automated sequencer. Each sample was processed with its corresponding nested primers. If a sample tested positive for both avian and mammalian markers in the PCR reaction, then two sequence products were obtained. Two independent reactions were carried out for each PCR product in order to obtain a forward and reverse strain . The conditions for the PCR were as recommended by the manufacturer (Appendix B). If a sample amplified both avian and mammalian fragments, then four cycle sequencing products were obtained.

In order to remove the unused dye terminators, the samples were purified using the BigDye XTerminator Purification kit according to the manufacturer's recommendations (Applied Biosystems, Appendix B). The purified DNA was then transferred to a sequencing plate and loaded into the 3500xL Genetic Analyzer (Applied Biosystems). Sequencing conditions were set using the 3500 Data Collection Software,

according to the expected fragment size and the cycle sequencing purification method applied (BigDye XTerminator and for 700 base pairs).

The resulting sequences F and R sequence strains were aligned to obtain a consensus per sample. They were manually screened for inconsistencies and amended for possible artifacts from the sequencing processing using SeqMan (DNASTAR Lasergene, Madison, WI). All sequences were then aligned and compared using BioEdit (Carlsbad, CA). If there were discrepancies or any ambiguity in the base pairs, the original electropherogram was checked once again to ensure accuracy. Individual sequences were then blasted for comparison to reference sequences published in GenBank, using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). When possible, each sequence was identified to the species level allowing for 5% base pair mismatch.

Species that were identified as plausible blood meal hosts using the following qualifications were then recorded: results had to match to the sequence available on GenBank with a greater than 95% base pair match, and the species had to be present and available in the state of Georgia. If the species was not a plausible mosquito host in Georgia or did not match with an acceptable percentage to sequences on GenBank, a comparison at the genus level was performed using avian 16S sequences obtained from serum of avian species captured within the study area (59). Sequence comparisons were performed using MEGA5 (60).

Quality Control

Quality control measures were implemented in order to verify the specificity of the applied methodology and to validate the high frequency of human blood meals.

a) Any PCR result with a trace of DNA in the negative control lane was discarded. If the reaction was performed using a human sample as a positive control, a second reaction was performed using an Eastern Gray Squirrel DNA as a positive control. The sample was then re-sequenced and results were confirmed or discarded if any inconsistency was found.

b) An independent PCR was done in a random subset of samples that yielded human as a blood meal result using a squirrel sample as positive control to confirm the results. All samples evaluated (seven out of seven) returned the same results, confirming the original human blood meals source and indicating that a contamination at the PCR level is unlikely.

c) The sequences from the 32 samples that matched with a human blood meal source were analyzed and compared in terms of nucleotide variability. The 32 samples yielded only three different haplotypes, indicating that there were at least three different human hosts, but this was not the haplotype diversity that was expected. Given the low variability observed within the target region and given that the positive control for the PCR reactions was from a human, an independent sequence target was evaluated in search for a different longer sequence region to compare the blood meal sources results.

A third of samples that tested positive for mammalian blood meals were then tested with primers targeting Cytochrome B (CytB), which is commonly used to identify mosquito blood meals to the species level (25, 31, 61, 62). The PCR product was run through a 1.0% agarose gel at 100V for 45 minutes. When multiple bands were observed in the gel, bands representing the size of the target fragment were purified from a gel using a QIAquick Gel Extraction Kit (Qiagen). A second PCR reaction was performed

using this purified amplification product as template. When a clean product was observed in the gel, the product was purified and underwent cycle sequencing. A sequence was obtained from two non-human blood meals, a common raccoon and a white-tailed deer. The majority of the other samples did not yield visible product for gel extraction and sequencing. Unfortunately, because of the low specificity of the technique, this process did not confirm nor deny the plausibility of the human blood meal sources.

d) A majority of the samples yielding human sequences that were identical with only three haplotypes, and many matched 100% to sequences on GenBank. These sequences, however, were approximately 16,500 base pairs long and extended beyond the 16S ribosomal DNA that was the targeted region of 550 base pairs. Although the reference sequences from GenBank matched completely (100%) to the haplotypes obtained in the samples, several nucleotide differences were observed outside the target region. This method has been previously used to detect the level of variation within a targeted region (49). Therefore, it was concluded that the 16S region used as target for the study is extremely conserved within species, thus making the target sequence undistinguishable even if the blood meals came from different humans.

Data processing and analysis

The results were entered into Excel (2011) for data management. Descriptive information of each mosquito was previously collected at the time of capture. Each experimental protocol performed and each subsequent result was recorded. Descriptive statistics were calculated after importation to SAS 9.3 (Cary, NC).

Dichotomized variables, such as creek and year, were tested for significance using a Fisher’s exact test was performed due to limited data. The month variable was

dichotomized in order to create season, a variable that represented the before and after the start of the WNV transmission season. The incidence of West Nile Virus increases considerably around mid-July and therefore the seasons were dichotomized as prior to the sudden increase, and after the increase (Figure 3).

In addition, a logistic regression was applied to the data to create a model that includes year, month, creek, and season. Stepwise elimination was used in order to determine a model with significant predictors. No interaction variables were included due to lack of evidence. All data analysis was performed using SAS 9.3 (Cary, NC).

Results

Mosquito collections, species, Sella scores

A total of 25,188 mosquitoes were caught at the two creeks in 2010 and 2011. 15,578 mosquitoes were collected in Tanyard Creek (61.8%) in eight sampling sites versus 9,610 in Peavine Creek (38.1%), in three sampling sites. In terms of years, 60 blood fed mosquitoes (70%) were caught in 2010, while 26 (30%)were captured in 2011. Differences in the number of mosquitoes caught per creek were insignificant (p=0.41). About 46% of the captured mosquitoes were *Culex pipiens quinquefasciatus*; 50.8% of the remaining mosquitoes were only identifiable to the genus *Culex spp.* (Table 2). On average, 0.60% of the mosquitoes were found to be blood fed. There were 96 mosquitoes with a Sella score between two and five, which represented mosquitoes with a fresh blood meal and mosquitoes with a partially digested blood meal, respectively (Table 2).

Blood meal analysis

Of 75 samples were successfully sequenced, a 69 blood meals were matched with a plausible species. In total, there were 37 (43%) blood meals of avian origin, 38 (44%) blood meals of mammalian origin, and five detected blood meals of amphibian origin (Table 3). Ten (27%) of the avian blood meals originated from a *Cardinalis cardinalis* (Northern Cardinal), and eight (22%) of the avian blood meals originated from an *Turdus migratorius* (American Robin). An unidentified avian species that matched best to a species in GenBank not found in Georgia (*Chrysomus thilius,* or Yellow-winged Blackbird) comprised of 22% of the avian sample. Lastly, two blood fed mosquitoes, or 5% of the blood meals, had fed on the *Sturnus vulgaris* (Common Starling). All other blood meals came from unique avian species, with each blood meal accounting for 3% of the total (Table 3). Within the mammalian samples, human blood meals were the most frequently detected, making up 84% of the total mammalian samples (Table 3).

Eight samples (9.3%) amplified for both avian and mammalian primers (Table 5). These were classified as mixed blood meals, meaning that the mosquito had fed on both an avian species and a mammalian species. Seven, or 8.1% of the total mosquitoes, contained mixed blood meals that identified human as the mammalian species (Table 5).

The percentage of avian blood meals ranged from 13% to 73% at the various sampling sites (Table 4). Overall, 61% of the blood fed mosquitoes obtained from Peavine Creek had fed on avian species while 34% of the blood fed mosquitoes from Tanyard Creek had fed on avian species (Table 4).

Analytic statistics

Avian host choice was significantly associated with creek, but not with year or month, while human host choice was associated with year and creek. The probability of feeding on an avian at Peavine Creek was 3.05 times than at Tanyard Creek (Fisher's exact test, $p=0.0127$) (Table 6, Figure 6) and the reverse was true for humans, with the probability of a mosquito having a human blood meal at Tanyard Creek being 3.21 times higher than at Peavine Creek (Fisher's exact test, $P=0.231$) (Table 6, Figure 6). In addition, there was a significant association between human feeding and year, with the probability of a mosquito feeding on a human in 2010 was 12 times higher than in 2011 (Fisher's exact test, $p=0.0002$) (Table 6, Figure 6). There was no significant relationship between American Robin blood meals and year, season, or creek.

Month, year, season, and creek were identified as potential predictors for mosquito host choice, comparing avian versus not avian blood meals, human versus not human blood meals, and American robin versus all avian blood meals, and were all assessed for significance. Creek was the only significant predictor for whether a mosquito fed on an avian blood meal, but only accounted for 7% of the variation in the outcome (multiple logistic regression, adjusted $r^2=0.06$, $p<0.05$). In addition, year was found to be the only significant predictor of human versus nonhuman blood meals, but did not account for much of the variance present (multiple logistic regression, adjusted $r^2=0.15$, $p<0.001$) (Table 7). Month and season were not statistically associated with any host preference in blood meals.

Discussion

In WNV, mosquito host preference can determine the frequency of several types of virus transmission- interspecies or intraspecies transmission, and the frequency of spillover. Ornithophilic mosquitoes will primarily transmit WNV between the various species of avian reservoir hosts, while opportunistic feeders may feed equally on both avians and mammals, acting as the bridge vector between taxonomic classes. The latter is the cause of public health concerns as humans may become infected. In 2002-2004, 7.8% of birds in Georgia were found to have antibodies to WNV (63), confirming the ongoing circulation of WNV within the avian system in the state.

Vector competence is typically classified by several factors- ability to be infected (infection rate) and ability to disseminate the virus (dissemination rate) (22, 64). A mosquito must be both a competent vector in field conditions and feed on both avian and mammalian species to transmit the pathogen (23). Feeding preferences of mosquito species and likelihood of virus transmission to mosquito have been found to vary over region and area. Even when exposed to identical experimental titers of WNV, populations of *Culex pipiens quinquefasciatus* were found to have different infection rates, highlighting the disparities within the geographically-distinctive populations (24). Characterization of mosquito feeding in the Atlanta area may yield to the contributing factors of why Atlanta has a low WNV incidence compared to similar urban areas, in addition to the community composition mentioned previously.

The feeding patterns of *Culex pipiens quinquefasciatus* in Atlanta's urban creeks yielded a greater percentage of human blood meals out of total blood meals than in other regions (28, 29, 31). This finding would suggest that WNV incidence rates should be

higher than in other areas due to the frequency a mosquito may feed on an avian and a human in the same lifetime. However, since WNV incidence rates are lower than in other states (Figure 1), there may be other factors that significantly contribute to the WNV transmission system. For example, there was found to be a significant association between capture site (creek) and percentage of avian blood meals, which can be associated with the vector-host community of each creek. In addition, the areas surrounding each creek are unique- Tanyard Creek is closer to the city of Atlanta and has more urban characteristics. Some sampling sites at Tanyard Creek are proximal to playgrounds, golf courses, and tennis courts. On the other hand, Peavine Creek is nested in a more suburban area, but are near baseball fields. Therefore, the surrounding areas of these two creeks may affect the host community in each of these locations.

The host community within each creek is important due to differences within avian species to spread WNV, which in turn leads to the dilution effect. Within the West Nile Virus transmission cycle, several avian hosts have been identified as species of interest- Northern Cardinals, American Robins, and House Sparrows. These avian hosts were identified to be competent hosts that can further transmit WNV(15, 24). In 2004, Northern Cardinals have been found to have 18.4% positive seroprevalence within the Piedmont region of Georgia, which includes Atlanta, highlighting their importance in the transmission of WNV (65).

Overall, this study supported the previous literature on *Culex spp.* feeding choice, as *Culex pipiens quinquefasciatus* has been shown to vary in both feeding preference and vector competence by region (66). The proportion of mosquitoes that fed on mammals was high, however, previous studies have yielded percentages ranging from 2.5%

mammalian blood meals to 65% mammalian blood meals (26-31). Therefore, percentage of mammalian blood meals (44%) observed in this study falls within the range of the literature. In addition, the percentage of avian blood meals was 43%, which fell within the literature range of 32% to 93.1% (26-31). The plethora of studies noted occurred in different locations from across the United States and varying ecological niches and therefore, some variation is expected within the data.

The majority of the avian blood meals came from three species of interest- Northern Cardinals, American Robins and House Sparrows. These three species have all been identified as competent reservoirs in a laboratory setting, based on susceptibility, mean infectiousness, and mean duration of illness (15, 67). American Robins and House Sparrows have also demonstrated virus amplification in the field, and its importance has been highlighted in the literature (27, 36). Therefore, the high prevalence of blood meals on these species may lead to an increase in the likelihood WNV cases at the mosquito-bird interface due to their ability transmit WNV. In addition, Northern Cardinal populations have been shown to have a high level of seroprevalence for WNV, with approximately 70% of Cardinals having been exposed to the virus in Georgia (65, 68, 69). This high seroprevalence is expected in these species due to the presence of ornithophilic mosquitoes and the high number of blood meals taken from these species. The direct comparison of avian feeding preference will vary significantly over geographic areas due to availability of certain avian hosts.

In this study, the majority, 84% of mammalian originated blood meals were identified as human. The measurement of human feeding is important in determining the frequency of human-mosquito interaction and therefore is critical in characterizing WNV

transmission. Past studies have shown varying amounts of human blood meals ranging from 2% of the mammalian blood meals to 83% of the mammalian blood meals (26-31). The proportion of humans in these urban creek sites is relatively high due to their proximities to parks, fields, trails, playgrounds and apartment complexes. Therefore, access to human hosts is plentiful and plausible. T1 and T2 had the highest percentages of human blood meals (2/2 blood meals, and 2/3 blood meals respectively). This result is possibly due to host availability, since T1 and T2 are close to an apartment complex, and therefore would be proximal to a large amount of potential human hosts.

One aspect of importance is the presence of mixed feeds, which imply that a mosquito has fed on both an avian species and a mammalian species. The presence of mixed feeds has many public health implications, as it would represent the interface between which WNV is transmitted to humans. Since the majority of the mixed feeds involved human blood, mixed feeds represent the interface where WNV may spillover into humans from the avian reservoir. However, it is important to note that using these methods, it is more likely to determine cross-class feeding. Identifying mixed feeds from hosts the same taxonomic class using traditional DNA sequencing methods is quite difficult due to technical impediments. If there were multiple blood meals from the same host class present, the PCR reaction would amplify two different products of the same length. If the amplified product of both blood meals sources is not separated before setting up the sequencing reaction, the resulting sequence product would carry ambiguities, and cannot be interpreted. Therefore, with these methods, possible transmission from avian-to-mammalian species may be determined, but avian-to-avian transmission cannot be detected.

In addition, the year of sampling was also found to be a significant predictor on the percentage human blood meals. This may be due to environmental and community factors; in 2010, Tanyard Creek underwent significant changes due to the opening of the Atlanta BeltLine. The Atlanta BeltLine is consists of newly created and renovated trails and parks in the Atlanta area and increased human traffic within the area. With the majority of the human blood meals originating from Tanyard Creek, it is hypothesized that there was a significant increase in human traffic through the areas of Tanyard Creek may be due to this new development. The differences in year may also be an artifact from the sample size of each year, as more mosquitoes were caught in 2010 than 2011 (70% of the mosquitoes analyzed were obtained in 2010). Lastly, the 2010 and 2011 blood fed mosquitoes were processed separately, and it is possible that there was human error during the processing or extraction process that led to these results.

The conclusion that the feeding preferences may be purely based upon creek and time suggests that *Culex pipiens quinquefasciatus* is a primarily opportunistic feeder that can shift from host to host. Other studies that show preferential feeding may reflect artifacts in seasonal shifts due to undetected community shifts in other mosquito preference studies. These results support the conclusion that *Culex pipiens quinquefasciatus* can contribute to the spillover of WNV into humans due to its feeding preferences.

Limitations

The limitations of the study may explain the discrepancies in conclusions. There are several categories of limitations identified with this process- molecular based, sequencing based, and analysis based.

During the mosquito processing steps, there is always potential for contamination from other sources. The human samples are difficult to confirm, as many people processed each mosquito. For example, in one of the samples, the mammalian primer amplified DNA from *Chlorocebus sabaeus*, a green monkey. It is hypothesized that during WNV testing, the green monkey kidney cells from the inoculum were introduced into the sample due to human error. However, it would be difficult to detect such an error with human DNA.

There are a limited number of sequences available in GenBank for the 16S ribosomal gene, which affected the results of the BLAST process. However, we obtained reference sequences from the serum of bird species present in Georgia, that were compared to the blood meal sequences that could not be determined by comparison of reference sequences from GB. One set of similar samples, hypothesized to be the same species, remained unmatched, but the majority of samples matched 99-100% to the corresponding serum sequences.

In addition, there is a lack of information regarding the bird community composition of the creeks. An assumption that the avian and mammalian communities stay constant throughout the season and the year had to be made to compare any feeding preferences. However, this was challenged with the changes to Tanyard Creek. Conclusions may still be drawn regarding the variety of hosts that *Culex spp.* have fed on.

There is also a limited sample size, which hindered more in depth analysis. As only 0.6% of the mosquitoes were blood fed, better methods could have been employed to target blood fed mosquito capture. Resting boxes have been found to increase yield of blood fed mosquitoes, but were difficult to implement due to logistical limitations (70,

71). In addition, due to the unpredictable nature of the urban creeks, resting boxes were occasionally washed away during periods of creek flooding. Other methods of increasing sample size include increasing the number of locations with gravid traps, increasing the number of creeks sampled, or increasing aspiration time.

Lastly, these conclusions may not be generalizable to the entire United States due to the differences in community composition of avian and mosquito species. However, the characterization of the feeding preference of *Culex pipiens quinquefasciatus* is necessary for further analyzing the transmission patterns of WNV within the United States.

Future Directions

The characterization of mosquito feeding choice is ongoing and developing. The use of the 16S marker has been found as an extremely sensitive method that is well suited for the lack of host DNA available when analyzing digested mosquito blood meals. The characterization of the *Culex* mosquitoes at the urban creeks is a novel idea that will benefit from more data. Mosquitoes from 2012 captures will be processed and included in the next analysis, optimally strengthening the associations detected in this preliminary study.

References

1. Jones KE, Patel NG, Levy MA, et al. Global trends in emerging infectious diseases. *Nature* 2008;451(7181):990-3.
2. Patz JA, Epstein PR, Burke TA, et al. Global climate change and emerging infectious diseases. *JAMA : the journal of the American Medical Association* 1996;275(3):217-23.
3. Morse SS. Factors in the emergence of infectious diseases. *Emerging infectious diseases* 1995;1(1):7-15.
4. Weiss RA, McMichael AJ. Social and environmental risk factors in the emergence of infectious diseases. *Nature medicine* 2004;10(12 Suppl):S70-6.
5. Chua KB. Nipah virus outbreak in Malaysia. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2003;26(3):265-75.
6. Greger M. The human/animal interface: emergence and resurgence of zoonotic infectious diseases. *Critical reviews in microbiology* 2007;33(4):243-99.
7. Chua KB, Bellini WJ, Rota PA, et al. Nipah virus: a recently emergent deadly paramyxovirus. *Science* 2000;288(5470):1432-5.
8. Alirol E, Getaz L, Stoll B, et al. Urbanisation and infectious diseases in a globalised world. *The Lancet infectious diseases* 2011;11(2):131-41.
9. Nash D, Mostashari F, Fine A, et al. The outbreak of West Nile virus infection in the New York City area in 1999. *The New England journal of medicine* 2001;344(24):1807-14.

10. Centers for Disease Control and Prevention. West Nile Virus. 2012. (http://www.cdc.gov/ncidod/dvbid/westnile/index.htm). (Accessed January 17 2013).

11. Campbell GL, Marfin AA, Lanciotti RS, et al. West Nile virus. *The Lancet infectious diseases* 2002;2(9):519-29.

12. Weiss D, Carr D, Kellachan J, et al. Clinical findings of West Nile virus infection in hospitalized patients, New York and New Jersey, 2000. *Emerging infectious diseases* 2001;7(4):654-8.

13. Schmidt KA, Ostfeld RS. Biodiversity and the dilution effect in disease ecology. *Ecology* 2001;82(3):609-19.

14. Ostfeld RS, Keesing F. Biodiversity and disease risk: The case of lyme disease. *Conservation Biology* 2000;14(3):722-8.

15. Komar N, Langevin S, Hinten S, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerging infectious diseases* 2003;9(3):311-22.

16. Allan BF, Langerhans RB, Ryberg WA, et al. Ecological correlates of risk and incidence of West Nile virus in the United States. *Oecologia* 2009;158(4):699-708.

17. Ezenwa VO, Godsey MS, King RJ, et al. Avian diversity and West Nile virus: testing associations between biodiversity and infectious disease risk. *Proceedings Biological sciences / The Royal Society* 2006;273(1582):109-17.

18. Swaddle JP, Calos SE. Increased avian diversity is associated with lower incidence of human West Nile infection: observation of the dilution effect. *PloS one* 2008;3(6):e2488.

19. Ostfeld RS, Keesing F. The function of biodiversity in the ecology of vector-borne zoonotic diseases. *Can J Zool* 2000;78:2061-78.

20. Randolph SE, Dobson AD. Pangloss revisited: a critique of the dilution effect and the biodiversity-buffers-disease paradigm. *Parasitology* 2012;139(7):847-63.

21. Dye C. Vectorial capacity: must we measure all its components? *Parasitol Today* 1986;2(8):203-9.

22. Sardelis MR, Turell MJ, Dohm DJ, et al. Vector competence of selected North American Culex and Coquillettidia mosquitoes for West Nile virus. *Emerging infectious diseases* 2001;7(6):1018-22.

23. Turell MJ, Dohm DJ, Sardelis MR, et al. An update on the potential of north American mosquitoes (Diptera: Culicidae) to transmit West Nile Virus. *J Med Entomol* 2005;42(1):57-62.

24. Reisen WK, Fang Y, Martinez VM. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. *J Med Entomol* 2005;42(3):367-75.

25. Kilpatrick AM, Daszak P, Jones MJ, et al. Host heterogeneity dominates West Nile virus transmission. *Proceedings of the Royal Society B: Biological Sciences* 2006;273(1599):2327-33.

26. Zinser M, Ramberg F, Willott E. Culex quinquefasciatus (Diptera: Culicidae) as a potential West Nile virus vector in Tucson, Arizona: blood meal analysis indicates feeding on both humans and birds. *J Insect Sci* 2004;4:20.

27. Hamer GL, Kitron UD, Goldberg TL, et al. Host selection by Culex pipiens mosquitoes and West Nile virus amplification. *The American journal of tropical medicine and hygiene* 2009;80(2):268-78.

28. Apperson CS, Hassan HK, Harrison BA, et al. Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. *Vector Borne Zoonotic Dis* 2004;4(1):71-82.

29. Molaei G, Cummings RF, Su T, et al. Vector-host interactions governing epidemiology of West Nile virus in Southern California. *The American journal of tropical medicine and hygiene* 2010;83(6):1269-82.

30. Molaei G, Andreadis TG, Armstrong PM, et al. Host feeding patterns of Culex mosquitoes and West Nile virus transmission, northeastern United States. *Emerging infectious diseases* 2006;12(3):468-74.

31. Garcia-Rejon JE, Blitvich BJ, Farfan-Ale JA, et al. Host-feeding preference of the mosquito, Culex quinquefasciatus, in Yucatan State, Mexico. *Journal of Insect Science* 2010;10(32).

32. Mboera LEG, W.Takken. Odour-mediated host preference of *Culex quinquefasciatus* in Tanzania. *Entomologia Experimentalis et Applicata* 1999;92:83-8.

33. Farid MA. Relationships between certain populations of Culex pipiens Linnaeus and Culex quinquefasciatus Say in the United States. *American journal of hygiene* 1949;49(1):83-100.

34. Sundararaman S. Biometrical studies on intergradation in the genitalia of certain populations of Culex pipiens and Culex quinquefasciatus in the United States. *American journal of hygiene* 1949;50(3):307-14.

35. Fonseca DM, Keyghobadi N, Malcolm CA, et al. Emerging vectors in the Culex pipiens complex. *Science* 2004;303(5663):1535-8.

36. Kilpatrick AM, Kramer LD, Jones MJ, et al. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS biology* 2006;4(4):e82.

37. Edman JD, Taylor DJ. Seasonal Shift in the Bird-Mammal Feeding Ratio in a Mosquito Vector of Human Encephalitis. *Science* 1968;161(3836):67-8.

38. Mackay AJ, Kramer WL, Meece JK, et al. Host feeding patterns of Culex mosquitoes (Diptera: Culicidae) in East Baton Rouge Parish, Louisiana. *J Med Entomol* 2010;47(2):238-48.

39. Bradley CA, Altizer S. Urbanization and the ecology of wildlife diseases. *Trends in ecology & evolution* 2007;22(2):95-102.

40. Brownstein JS, Rosen H, Purdy D, et al. Spatial analysis of West Nile virus: rapid risk assessment of an introduced vector-borne zoonosis. *Vector Borne Zoonotic Dis* 2002;2(3):157-64.

41. Brown HE, Childs JE, Diuk-Wasser MA, et al. Ecological factors associated with West Nile virus transmission, northeastern United States. *Emerging infectious diseases* 2008;14(10):1539-45.

42. Nowak DJ, Noble MH, Sisinni SM, et al. People and trees: Assessing the US urban forest resource. *Journal of Forestry* 2001;99(3):37-42.

43. Nowak DJ, Rowntree RA, McPherson EG, et al. Measuring and analyzing urban tree cover.pdf. *Landscape and Urban Planning* 1996;36:49-57.

44. King WV, Bull CG. The blood feeding habits of malaria-carrying mosquitoes. *American Journal of Epidemiology* 1923;3(5):497-513.

45. Beier JC, Perkins PV, Wirtz RA, et al. Bloodmeal identification by direct enzyme-linked immunosorbent assay (ELISA), tested on Anopheles (Diptera: Culicidae) in Kenya. *J Med Entomol* 1988;25(1):9-16.

46. Townzen JS, Brower AV, Judd DD. Identification of mosquito bloodmeals using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences. *Med Vet Entomol* 2008;22(4):386-93.

47. Lee JH, Hassan H, Hill G, et al. Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. *The American journal of tropical medicine and hygiene* 2002;66(5):599-604.

48. Terenius O, de Oliveira CD, Pinheiro WD, et al. 16S rRNA gene sequences from bacteria associated with adult Anopheles darlingi (Diptera: Culicidae) mosquitoes. *J Med Entomol* 2008;45(1):172-5.

49. Szalanski AL, Tripodi AD, Austin JW. Multiplex polymerase chain reaction diagnostics of bed bug (Hemiptera: Cimicidae). *J Med Entomol* 2011;48(4):937-40.

50. Jones CM, Nagel L, Hughes GL, et al. Host specificity of two species of Gnathia (Isopoda) determined by DNA sequencing blood meals. *International journal for parasitology* 2007;37(8-9):927-35.

51. Vences M, Thomas M, van der Meijden A, et al. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Front Zool* 2005;2(1):5.

52. Killmaster LF, Stallknecht DE, Howerth EW, et al. Apparent disappearance of Vesicular Stomatitis New Jersey Virus from Ossabaw Island, Georgia. *Vector Borne Zoonotic Dis* 2011;11(5):559-65.

53. Vazquez-Prokopec GM, Galvin WA, Kelly R, et al. A new, cost-effective, battery-powered aspirator for adult mosquito collections. *J Med Entomol* 2009;46(6):1256-9.

54. Maia MF, Robinson A, John A, et al. Comparison of the CDC Backpack aspirator and the Prokopack aspirator for sampling indoor- and outdoor-resting mosquitoes in southern Tanzania. *Parasites & vectors* 2011;4:124.

55. Jr RFD, Ward RA, Carpenter SJ, et al. *Identification and geographical distribution of the mosquitoes of North America, north of Mexico.* 2nd edition ed. Gainesville, FL: University of Florida Press; 2004.

56. Barr AR. The distribution of Culex p. pipiens and C.P. quinquefasciatus in North America. *The American journal of tropical medicine and hygiene* 1957;6(1):153-65.

57. Detinova TS. *Age Grouping Methods in Díptera of Medical Importance with special reference to some Vectors of Malaria*. World Health Organization Monograph; 1962.

58. Martinez-de la Puente J, Ruiz S, Soriguer R, et al. Effect of blood meal digestion and DNA extraction protocol on the success of blood meal source determination in the malaria vector Anopheles atroparvus. *Malaria journal* 2013;12:109.

59. Levine RCS. Unpublished data. Emory University, 2013.

60. Tamura K, Peterson D, Peterson N, et al. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* 2011;28:2732-9.

61. Barrera R, Amador M, Young G, et al. Mosquito (Diptera: Culicidae) Bloodmeal Sources during a Period of West Nile Virus Transmission in Puerto Rico. *Journal of Medical Entomology* 2011;48(3):701-4.

62. Lura T, Cummings R, Velten R, et al. Host (avian) biting preference of southern California Culex mosquitoes (Diptera: Culicidae). *J Med Entomol* 2012;49(3):687-96.

63. Gibbs SE, Wimberly MC, Madden M, et al. Factors affecting the geographic distribution of West Nile virus in Georgia, USA: 2002-2004. *Vector Borne Zoonotic Dis* 2006;6(1):73-82.

64. Turell MJ, O'Guinn ML, Dohm DJ, et al. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. *J Med Entomol* 2001;38(2):130-4.

65. Gibbs SE, Allison AB, Yabsley MJ, et al. West Nile virus antibodies in avian species of Georgia, USA: 2000-2004. *Vector Borne Zoonotic Dis* 2006;6(1):57-72.

66. Goddard LB, Roth AE, Reisen WK, et al. Vector competence of California mosquitoes for West Nile virus. *Emerging infectious diseases* 2002;8(12):1385-91.

67. Komar N, Panella NA, Langevin SA, et al. Avian hosts for West Nile virus in St. Tammany Parish, Louisiana, 2002. *The American journal of tropical medicine and hygiene* 2005;73(6):1031-7.

68. Komar N, Burns J, Dean C, et al. Serologic evidence for West Nile virus infection in birds in Staten Island, New York, after an outbreak in 2000. *Vector Borne Zoonotic Dis* 2001;1(3):191-6.

69. Godsey MS, Jr., Blackmore MS, Panella NA, et al. West Nile virus epizootiology in the southeastern United States, 2001. *Vector Borne Zoonotic Dis* 2005;5(1):82-9.

70. Barrera R, Amador M, Young G, et al. Mosquito (Diptera: Culicidae) bloodmeal sources during a period of West Nile virus transmission in Puerto Rico. *J Med Entomol* 2011;48(3):701-4.

71. Burkett-Cadena ND, Eubanks MD, Unnasch TR. Preference of female mosquitoes for natural and artificial resting sites. *Journal of the American Mosquito Control Association* 2008;24(2):228-35.

Tables

Table 1. Primers used for blood meal analysis. Killmaster et al.2011 (1).

16S Primer name	Primer sequence 5’ to 3’
Vertebrate Forward	ACC CNT CYM TGT NGC AAA AKR GT
Avian Forward	MMC AAG TAT TGA AGG TGA
Avian Reverse	CTG ATC CAA CAT CGA
Mammalian Forward	CCT GTT TAC CAA AAA CAT CAC
Mammalian Reverse	AYT GTC GAT AKG RAC TCT WRA RTA G GGT CGT G

Table 2. Descriptive statistics of captured mosquitoes caught in Atlanta, Georgia 2010-2011

	Creek		
	Peavine	**Tanyard**	**Total**
Total caught	9610	15578	25188
Genus and species			
Culex unidentified	6040 (62.9)	6745 (43.3)	12785 (50.8)
Culex pipiens quinquefasciatus	3354 (34.9)	8335 (53.5)	11689 (46.4)
Culex restuans	167 (1.73)	380 (2.43)	547 (2.17)
Culex territans	7 (0.07)	28 (0.17)	35(0.14)
Culex salinarius	0 (0.00)	22 (0.14)	22 (0.09)
Culex erraticus	1 (0.01)	8 (0.05)	9 (0.04)
Culex nigropalpus	0 (0.00)	3 (0.01)	3 (0.01)
Aedes albopictus	22 (0.23)	7 (0.04)	29 (0.12)
Aedes vexans	3 (0.03)	11 (0.07)	14 (0.06)
Other			
Uranotaenia sappharina	0 (0.00)	9 (0.06)	9 (0.04)
Onchlerotatus triseritaus	3 (0.03)	0 (0.00)	3 (0.01)
Anopheles punctipennis	0 (0.00)	1 (0.01)	1 (0.00)
Anopheles quadrimaculitus	0 (0.00)	1 (0.01)	1 (0.00)
Sella score			
0	9544 (99.31)	15477 (99.35)	25021 (99.3)
1	3 (0.03)	11 (0.07)	14 (0.06)
2	5 (0.05)	3 (0.02)	8 (0.03)
3	2 (0.02)	4 (0.03)	6 (0.02)
4	9 (0.09)	26 (0.17)	35 (0.14)
5	24 (0.25)	23 (0.15)	47 (0.19)
6	20 (0.21)	34 (0.22)	54 (0.21)
Total bloodfed, n(%)	20 (0.21)	90 (0.58)	150 (0.60)

*Missing Sella score for 2 Peavine mosquitoes and 1 Tanyard mosquito
*Missing Sella score and creek for 2 mosquitoes
*No species classification for 12 Peavine, 8 Tanyard mosquitoes

Table 3. Blood meal analysis of mosquitoes captured at Peavine and Tanyard Creek 2010-2011.

Species	Common name	Blood meals	% of group	% of total (n=86)
Avian				
Cardinalis cardinalis	Northern Cardinal	9	24%	10%
*Chrysomus thilius**	*Blackbird*	8	22%	9%
Turdus migratorius	American Robin	8	22%	9%
Sturnus vulgaris	Common Starling	2	5%	2%
Dumetella carolinensis	Gray Catbird	1	3%	1%
Troglodytes aedon	House Wren	1	3%	1%
Baeolophus bicolor	Tufted Titmouse	1	3%	1%
Accipiter cooperii	Cooper's Hawk	1	3%	1%
Hylocichla mustelina	Wood Thrush	1	3%	1%
Carpodacus mexicanus	House Finch	1	3%	1%
Meleagris gallopavo	Wild Turkey	1	3%	1%
Poecile carolinensis	Carolina Chickadee	1	3%	1%
Gallus gallus	Chicken	1	3%	1%
Toxostoma rufum	Brown Thrasher	1	3%	1%
Total Avian		37	100%	43%
Mammalian				
Homo sapiens	Humans	32	84%	37%
Sciurus carolinensis	Eastern Gray Squirrel	1	3%	1%
Lasiurus borealis	Eastern Red Bat	1	3%	1%
Procyon lotor	Common raccoon	1	3%	1%
Odocoileus virginianus	White-tailed Deer	1	3%	1%
Didelphis virgiana	Virginia Oppossum	1	3%	1%
Canis lupus familiaris	Dog	1	3%	1%
Total Mammalian		38	100%	44%
Amphibian				
Rana clamitans	Green frog	4	80%	5%
Rana catesbeiana	American bullfrog	1	20%	1%
Total Amphibian		5	100%	6%

* Sequences matched over >95% to *Chrysomus thilius* species, but it is not found in Georgia

- 12 samples excluded from analysis. 11 negative for both Avian and Mammalian, 1 degraded, 1 contaminated with Green Monkey cells, 1 not found on GenBank

- 8 samples were positive for two species (one avian and one mammalian). See Table 5

Table 4. Sampling Locations in Atlanta, Georgia 2010-2011.

Location	Longitude	Latitude	Captured, n	# Blood fed, n(%)	Avian blood meals, n(%)	Human blood meals, n(%)
Peavine Creek						
P1	84°19'42.095"W	33°47'25.794"N	2999	6 (0.20)	3 (50)	3 (50)
P2	84°19'49.863"W	33°47'46.019"N	3925	15 (0.38)	8 (53)	3 (20)
P3	84°19'50.415"W	33°47'49.166"N	2686	15 (0.56)	11 (73)	2 (13)
Total			9610	36 (0.37)	22 (61)	0 (0)
Tanyard Creek						
T1	84°24'3.448"W	33°48'18.1"N	1273	2 (0.16)	1 (50)	2 (100)
T2	84°24'2.281"W	33°48'22.004"N	2875	5 (0.17)	2 (40)	3 (60)
T3	84°24'4.972"W	33°48'23.801"N	2074	11 (0.53)	4 (36)	3 (27)
T4	84°24'10.752"W	33°48'34.321"N	2584	7 (0.27)	2 (29)	3 (43)
T5	84°24'8.637"W	33°48'37.38"N	1570	8 (0.51)	1 (13)	4 (50)
T6	84°24'5.394"W	33°48'38.474"N	1142	7 (0.61)	3 (43)	4 (57)
T7	84°24'11.083"W	33°48'47.199"N	765	0 (----)	0 (- -)	0 (- -)
T8	84°24'15.557"W	33°48'54.131"N	3295	10 (0.30)	4 (40)	5 (50)
Total			15578	50 (0.32)	17 (34)	32 (64)

Table 5. Mixed blood meals in mosquitoes caught in Atlanta, Georgia 2010-2011

Avian species	Mammalian species	Mosquitoes, n
Dumetella carolinensis	*Lasiurus borealis*	1
Cardinalis cardinalis	*Homo sapiens*	3
*Chrysomus thilius**	*Homo sapiens*	2
Meleagris gallopavo[$]	*Homo sapiens*	1
Turdus migratorius	*Homo sapiens*	1

* Sequences matched over >95% to *Chrysomus thilius* species, but it is not found in Georgia

[$] The *Meleagris gallopavo* sequence was shorter than other matched sequences- 171/172 nucleotides

Table 6. Statistical analysis of dichotomized variables, comparing year, season, and creek, to overall feeding preference in Atlanta, GA 2010-2011

	Year		**Season[$]**		**Creek**	
	OR	**P-value**	**OR**	**P-value**	**OR**	**P-value**
Avian vs not Avian (n=60) (ref=not Avian)	0.39	0.0606	1.23	0.6628	3.05	0.0163
Human vs nonhuman (n=86) (ref=not human)	12.00	0.0002	0.61	0.3620	0.31	0.0231
American robin vs not American Robin (n=86) (ref=not Robin)	1.20	1.0000	0.83	1.0000	0.72	0.7089

[$] Season is dichotomized as before June 30th, or after June 30th

Table 7. P-values of the predictors included in the logistic regression.

	Variable			
Outcome	**Month**	**Year**	**Season**	**Creek**
Avian	0.1761	0.2529	0.4910	0.0124
Human	0.3240	0.0001	0.3157	0.1500
American Robin	0.8918	0.7439	0.9556	0.6290

Figures and Graphs

Figure 1. Cases of neuroinvasive WNV per 100,000 persons in the United States by state, 2009-2012. Data obtained from the Centers for Disease Control and Prevention (2) and the United States Census Bureau (3).

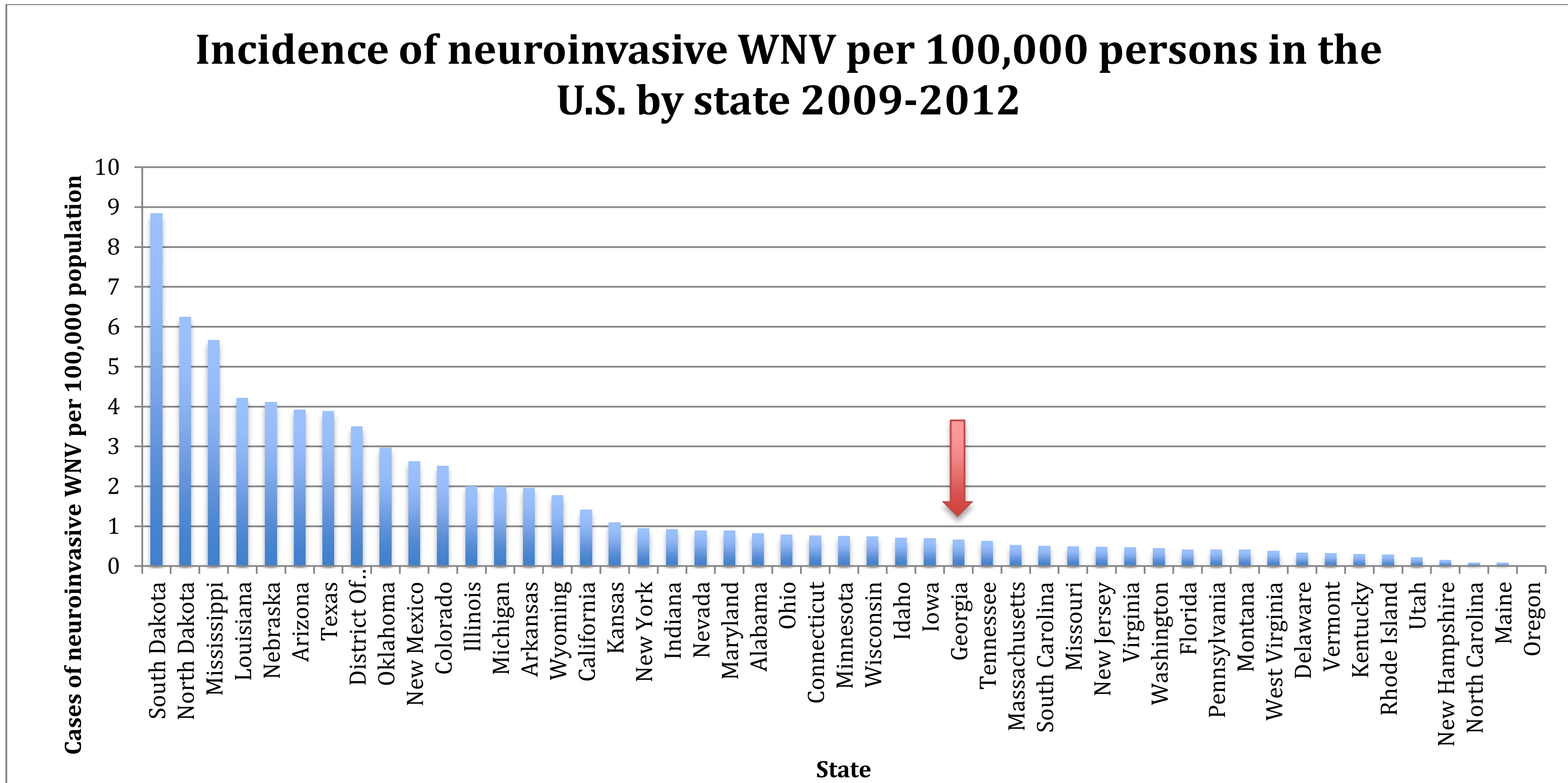

Figure 2. Map of sampling sites along Peavine and Tanyard Creek in Atlanta, GA 2010-2011. Adapted from Lund et al. (4).

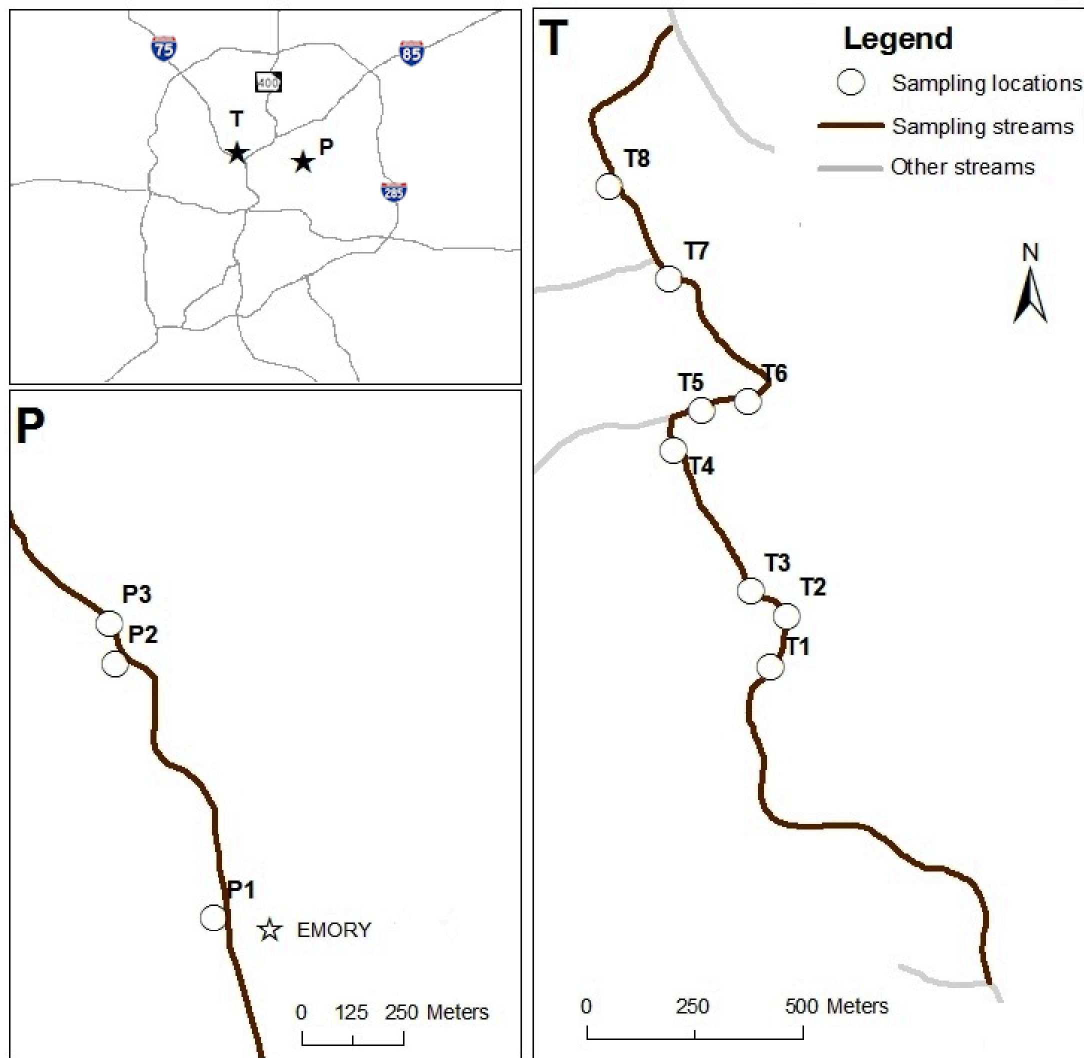

Figure 3. Epidemic curve of human disease due to WNV in the state of Georgia, 2010-2012. Obtained from ArboNet (2).

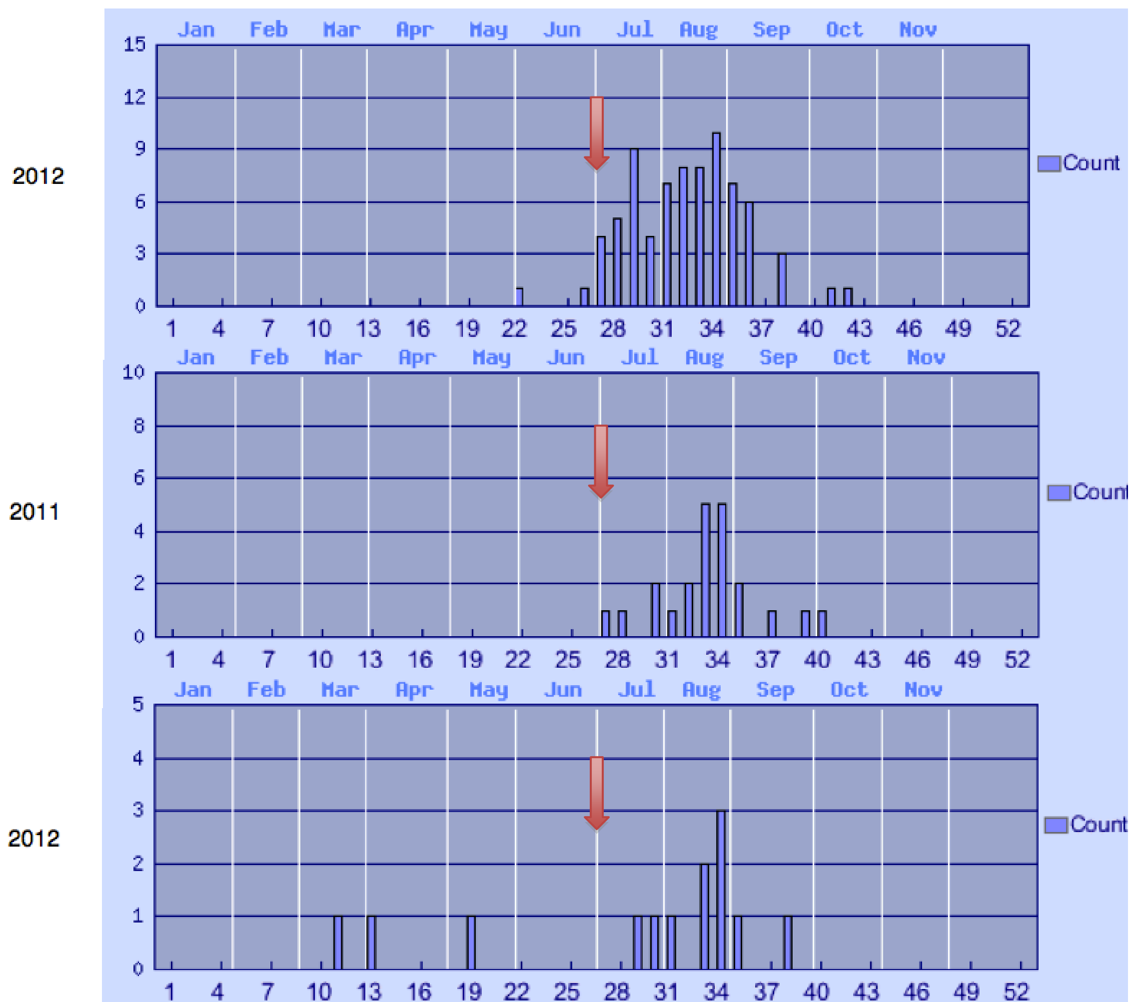

Figure 4. ***Culex pipiens*** **female mosquitoes with Sella score 2-6. As published by Martínez-de la Puente et al. (5)**

Figure 5. Blood meal analysis results by month, Atlanta, Georgia, 2010-2011

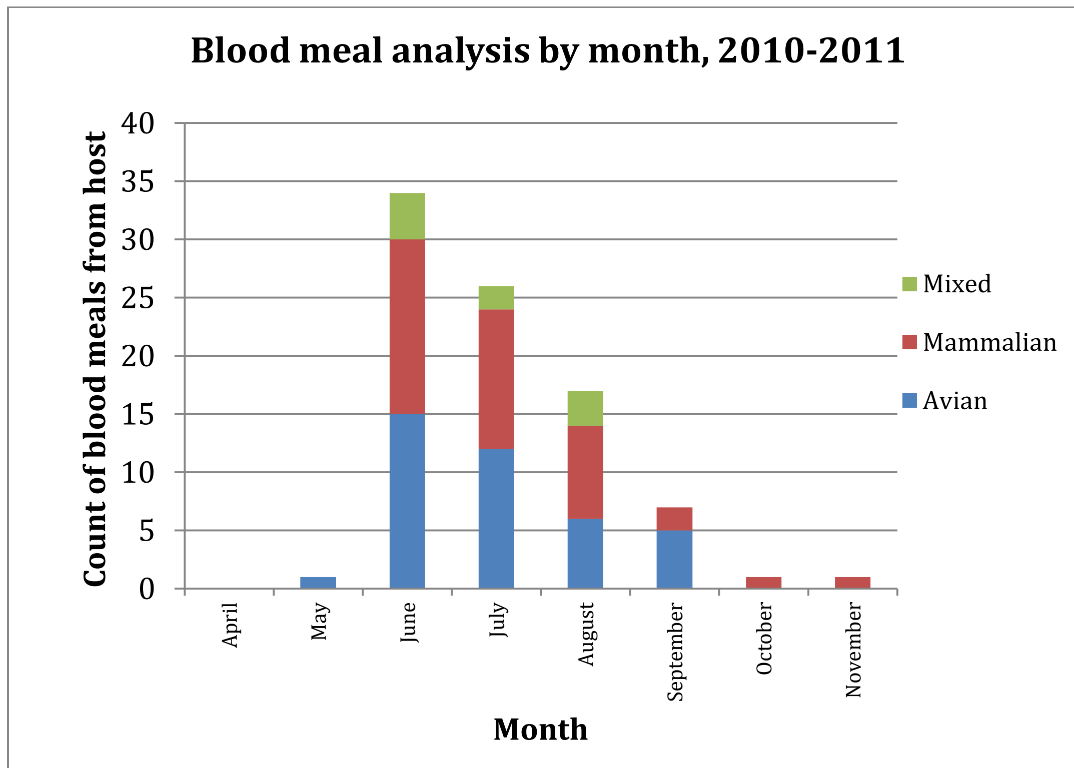

Figure 6. Statistical analysis of dichotomized variables, comparing year, season, and creek, to overall feeding preference in Atlanta, GA 2010-2011

References for Tables and Graphs

1. Killmaster LF, Stallknecht DE, Howerth EW, et al. Apparent disappearance of Vesicular Stomatitis New Jersey Virus from Ossabaw Island, Georgia. *Vector Borne Zoonotic Dis* 2011;11(5):559-65
2. Centers for Disease Control and Prevention. West Nile Virus. 2012. (http://www.cdc.gov/ncidod/dvbid/westnile/index.htm). (Accessed January 17 2013)
3. U.S. Census Bureau Population Division. Annual Estimates of the Population for the United States, Regions, States, and Puerto Rico: April 1, 2010 to July 1, 2012. 2012. (Accessed)
4. Lund A, McMillan JR, Kelly R, et al. Long term impacts of Combined Sewer Overflow remediation on water quality, mosquito population dynamics and West Nile virus amplification. (Manuscript submitted for publication).
5. Martinez-de la Puente J, Ruiz S, Soriguer R, et al. Effect of blood meal digestion and DNA extraction protocol on the success of blood meal source determination in the malaria vector Anopheles atroparvus. *Malaria journal* 2013;12:109

Appendix A: Laboratory protocols

Hemi-nested PCR protocol

Note: All PCR protocols should take place in a clean area (PCR hood or a biosafety hood) to reduce chances of contamination. Bleach and ethanol should be used to wipe all surfaces down, including pipettes.

Reagent list for master mix of hemi-nested PCR for blood meal analysis.

Reaction 1 Reagents	Amount (µl)	Reaction 2 Reagents	Amount (µl)
Avian master mix			
H_2O	12.4	H_2O	13.9
Buffer 5X (Promega)	5	Buffer 5X (Promega)	5
dNTPs (2.5mM)	2	dNTPs (2.5mM)	2
16SvertU_F	1	16Savian_F	1
16Savian_R	1	16Savian_R	1
MgCl2 (25mM)	1.5	MgCl2 (25mM)	1
taq	0.15	taq	0.15
Mammalian master mix			
H_2O	11.9	H_2O	12.4
Buffer 5X (Promega)	5	Buffer 5X (Promega)	5
dNTPs (2.5mM)	2	dNTPs (2.5mM)	2
16SvertU_F	1	16Smammalian_F	1
16Smammalian_R	1	16Smammalian_R	1
MgCl2 (25mM)	2.0	MgCl2 (25mM)	2
taq	0.15	taq	0.15

First PCR reaction

1. Label Eppendorf tubes for master mix. Must have two if running avian and mammalian PCRs.
2. For an Avian reaction, add in the following per sample: 12.4 µl of H2O, 5 µl of 5X Colorless GoTaq® Reaction Buffer, 2.0 µl of 2.5mM dNTPs, 1.0 µl of the forward vertebrate primer, 1.0 µl of the reverse avian primer, 1.5 µl of $MgCl_2$ (25mM), and 0.15 µl of Taq polymerase.

3. For a mammalian reaction, add in the following per sample: 11.9 µl of H2O, 5 µl of 5X Colorless GoTaq® Reaction Buffer, 2.0 µl of 2.5mM dNTPs, 1.0 µl of the forward vertebrate primer, 1.0 µl of the reverse mammalian primer, 2.0 µl of $MgCl_2$ (25mM), and 0.15 µl of Taq polymerase.
4. Mix the master mix by pulsing the sample on a vortex.
5. Distribute 23 µl of the master mix to each PCR tube.
6. Add in 2 µl of your sample.
7. Briefly spin down the tube(s) on a [small centrifuge] to get the liquids on the sides of the tube.
8. Program the BIO-RAD thermocycler (BIO-RAD, Hercules, CA) for the following conditions: 1 cycle of denaturation at 93°C for 2 minutes 30 sec, 39 cycles of denaturation (93°C for 30 seconds), annealing (50°C for 30 seconds), and extension (72°C for 1 minute), and a final extension step of 72°C for 10 minutes.
9. Store sample at 4°C or continue to 2nd PCR reaction.

Second PCR reaction

1. Label Eppendorf tubes for master mix. Must have two if running avian and mammalian PCRs.
2. For an Avian reaction, add in the following per sample: 13.9 µl of H2O, 5 µl of 5X Colorless GoTaq® Reaction Buffer, 2.0 µl of 2.5mM dNTPs, 1.0 µl of the forward avian primer, 1.0 µl of the reverse avian primer, 1.0 µl of $MgCl_2$ (25mM), and 0.15 µl of Taq polymerase.
3. For a mammalian reaction, add in the following per sample: 13.4 µl of H2O, 5 µl of 5X Colorless GoTaq® Reaction Buffer, 2.0 µl of 2.5mM dNTPs, 1.0 µl of the

forward vertebrate primer, 1.0 µl of the reverse mammalian primer, 1.5 µl of $MgCl_2$ (25mM), and 0.15 µl of Taq polymerase.

4. Mix the master mix by pulsing the sample on a vortex.
5. Distribute 24 µl of the master mix to each PCR tube.
6. Add in 1 µl of the product from the first reaction.
7. Briefly spin down the tube(s) in a centrifuge to get the liquids on the sides of the tube.
8. Program the BIO-RAD thermocycler (BIO-RAD, Hercules, CA) for the following conditions: 1 cycle of denaturation at 93°C for 2 minutes 30 sec, 39 cycles of denaturation (93°C for 30 seconds), annealing (52°C for 30 seconds), and extension (72°C for 1 minute), and a final extension step of 72°C for 10 minutes.
9. Start the run.
10. Store sample at 4°C.

Gel electrophoresis

1. Pour a 1.5% agarose gel with ethidium bromide.
2. Wait 45 minutes to cool.
3. Place gel into gel box (Thermo Scientific)
4. Pour in TAE buffer until gel is covered.
5. Load 5 µl of ladder.
6. Load 9 µl of each sample
7. Run at 120V for 45 minutes, or until the blue front is ¾ down the gel.

8. Visualize using UV methods- EpiChem3 Darkroom and Labworks (UVP LLC, Upland, CA)

Purification using Multiscreen PCR Plate (Millipore)

1. Load all of the PCR product into one well of the plate.
2. Turn vacuum on and wait for liquid to dissipate (1-2 minutes)
3. After all the liquid has left the sample, add 80 µl of nuclease-free water.
4. Turn vacuum on and wait for liquid to dissipate (3-4 minutes).
5. After all the liquid has left the sample, add 20 µl of Tris-HCL (TE buffer 1X). If needed, adjust TE amount to band intensity.
6. Shake on Clinical Rotator (Fisher Scientific) for 15 minutes on the 110 setting.
7. Transfer to 48-well PCR plate for storage at -20°C.

Cycle sequencing

1. Label PCR tubes.
2. Make master mix, one for each primer by adding in the following per sample: 5 µl of H2O, 2 µl of ABI 5X, 1 µl of primer (forward avian, reverse avian, forward mammalian, or reverse avian), and 0.5 µl of BDv1.1.
3. Program the BIO-RAD thermocycler (BIO-RAD, Hercules, CA) for the following conditions: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.
4. Start the run.
5. Store at 4°C in the dark for less than 24 hours.

Purification of cycle sequencing product

Adapted from the BigDye XTerminator Purification Kit Protocol

1. Calculate the following needed: 45 µl per sample of SAM, and 10 µl per sample of XTerminator. Multiply the amounts by 1.1 in order to have 10% extra volume.
2. Vortex the XTerminator solution until it is homogeneous (10 seconds).
3. Using a wide-bore pipette, extract the previously calculated amount of XTerminator.
4. Combine with the proper amount of SAM (also previously calculated)
5. Vortex the mixture until homogeneous.
6. Add in 55 µl of the SAM-XTerminator mixture into each sample. Agitate the SAM-XTerminator mixture before adding to ensure even mixture.
7. Put onto a vortex (2.5 setting) for 35 minutes.
8. Spin the samples down at 4000 rpm for two minutes.
9. Transfer 60 µl to sequencing plate.
10. If there are any remaining empty wells in a column, add HiDi™ formamide.

Sequencing

1. Spin down the samples at 4000 rpm for two minutes.
2. Place the plates into the adapter.
3. Load the plate apparatus into the 3500xL.
4. Use the BigDye XTerminator (BDx) fast setting for 700 bp.