THE FEEDING BIOLOGY OF THE TIGER MOSQUITO, *AEDES ALBOPICTUS*:
Bridging Ecology and Behavior to Identify Drivers of Blood and Sugar Feeding Patterns

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Abstract

Mosquitoes have shaped the natural world and human history through the pathogens that they transmit. Yet, despite millennia of cohabitation with these deadly insects, there is much that remains a mystery about them. In my dissertation, I seek to elucidate the complicated dynamics that drive mosquito feeding biology, including both blood and sugar feeding behavior and ecology. As my model organism to examine these questions, I worked with the tiger mosquito, *Aedes albopictus*, which can transmit over 20 different pathogens. The incredible invasive potential of *Ae. albopictus* has allowed it to establish throughout much of the world, leaving many people at risk of contracting disease. Its widespread threat to global health underscores the importance of researching the unanswered and under-studied aspects of its feeding biology critical to its vectorial capacity and life history.

In my first chapter, I reviewed the mosquito blood feeding literature, highlighting the distinction between host preference (the innate tendency of a mosquito species to choose one host species or group over others) and feeding patterns (the host usage in the field, influenced by both preference and environmental factors). There are numerous ways to assess preference and feeding patterns, which I described alongside the potential biases that these methods may introduce. Finally, I used *Ae. albopictus* as a case study to scrutinize the interpretation of feeding patterns and host preference. It serves as an ideal example to demonstrate the limitations of the available data and common obstacles to accurate interpretation. A combination of inconsistency

in the interpretation of blood meal analyses and a dearth of host preference research has resulted in conflicting descriptions of *Ae. albopictus* in the literature. The gaps in the literature identified in this chapter lay the foundation for the following two chapters.

The second chapter describes the feeding patterns of *Ae. albopictus* in Long Island, New York, based on a blood meal analysis of mosquitoes collected across several farms and residential neighborhoods. Blood fed mosquito collections were conducted in tandem with two host availability measurements – household interviews and camera traps. These data were used to calculate two feeding metrics, forage ratios and host feeding indices, providing more context for feeding pattern results. I found that *Ae. albopictus* fed on ten host species in New York and that it under-utilized humans compared with dogs and cats according to both time and abundance-weighted host feeding indices. Forage ratios also revealed over-utilization of cats and opossums and under-utilization of birds, squirrels, and raccoons.

Next, I conducted a life table analysis to assess the impact of host species on mosquito fitness to understand if certain host species provide a fitness advantage, which can serve as an evolutionary pressure to select for host preference. We fed New York *Ae. albopictus* blood from human, cat, opossum, horse, or rat and individually measured survival and fecundity. We then compared the fitness of *Ae. albopictus* from New York and Maryland, which have strikingly different feeding patterns, to assess whether differences in feeding patterns may be driven by differential impact of host species on fitness between the two populations. We did not find any major fitness differences by host species, indicating that underlying differences in fitness did not drive the observed feeding patterns. However, this finding did not rule out the possibility of variation in host preference between *Ae. albopictus* populations, which may exist between other

populations in the world or between the populations studied here, but driven by different evolutionary forces.

In chapter three, I directly assessed *Ae. albopictus* host preference. I included six populations from around the world, three with previously reported high levels of anthropophagy (human feeding) and three with low levels of anthropophagy, to assess the hypothesis that underlying differences in host preference drive the divergent feeding patterns. We used a dual-port olfactometer to present the mosquitoes with human and guinea pig odors and measured the host odor preference of each population. We compared the six *Ae. albopictus* populations to one another and to previously characterized anthropophilic and zoophilic *Ae. aegypti* colonies. We did not find differences in host preference between the *Ae. albopictus* populations, indicating that there is little variation for this trait and that the divergent feeding patterns were more likely the result of environmental factors, such as host availability. We also found that *Ae. albopictus* populations were less likely to choose human odor than the anthrophilic *Ae. aegypti* and behaved similarly to the zoophilic *Ae. aegypti.* This provided the first direct comparison of the host preference of these two vector species, which have overlapping ecologies and vector competencies.

In the fourth and final chapter, we again investigated the feeding patterns of *Ae. albopictus*, but with a focus on sugar rather than blood. Male mosquitoes rely strictly on sugar sources for nutrition and energy, whereas female mosquitoes utilize sugar to supplement nutrition from blood meals. The sugar feeding behavior of *Ae. albopictus* is poorly characterized; prior to this chapter, only three studies had measured sugar feeding in the field. In this study, I used a cold anthrone assay to determine the presence and concentration of fructose in *Ae. albopictus* collected in Long Island. In tandem with these collections, we measured the

temperature, humidity, and presence of flowers to understand the influence of these environmental parameters on sugar feeding. We collected both resting and host seeking mosquitoes, which provided information regarding the relationship between sugar feeding and host seeking in the field. We found that nearly half of *Ae. albopictus* were sugar fed and that each of the environmental and mosquito parameters that were measured had an impact on either the probability of sugar feeding or the concentration of fructose among sugar fed mosquitoes.

Together, the chapters in my dissertation advance our understanding of *Ae. albopictus* feeding biology, which is critical to predicting whether this invasive species may serve as a vector for the numerous pathogens that it can transmit. The identification and exclusion of certain drivers of feeding patterns through these studies can provide insights into how to harness the blood and sugar feeding behaviors for *Ae. albopictus* vector control.

Dedication

I dedicate this dissertation to my parents, Margaret and Erol Fikrig, who have supported and encouraged me in all my pursuits and fostered my love of bugs since I was a young kid, when I first decided I wanted to become an “antamologist”. Thank you for giving me my education, inspiring my curiosity, instilling in me a love of nature, and for opening so many doors for me and allowing me the freedom to choose which ones to take.

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To my former research mentors, teachers, and entomologist models, thank you for helping to guide me on my journey from a general interest in biology and bugs to becoming Ph.D. student. This dissertation would not exist without your influence on my life: My MPH advisor, Durland Fish, who first introduced me to the world of mosquito research; Scott Ritchie and Brian Johnson, who guided me through my first experience conducting a laboratory experiment with mosquitoes; Katelynn Mannix and Lynn Cooley for helping me to develop molecular research skills; my first research mentor, Don Windsor, who introduced me to tropical biology; my first entomology professor, Marta Wells, whose genuine and deep excitement for insects was incredibly infectious; my undergraduate advisor, Steven Stearns, who helped instill in me the knowledge that scientists are more than their science and whose encouragement was pivotal in my decision to take a year off to work on horse ranches before I started my Ph.D.; my AP Biology teacher, Ms. Heckman, who primed my interest to pursue biology in college; and Hannah Gould, who first taught me how to catch insects in my yard and whom I credit with my childhood aspiration to become an entomologist.

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Biographical Sketch

Kara Mackenzie Fikrig was born in New Haven, Connecticut in 1992. Her parents, Margaret and Erol Fikrig, raised her from a young age to love the outdoors and to be curious about the world around her. They have always supported all her interests and passions, not the least of which was entomology. Kara first learned about insects from an entomologist who showed her how to find and catch insects in her backyard. She began collecting cicada exoskeletons and stored them in unexpected places around the house and car – despite these habits, which some may have considered objectionable, her parents never wavered in their support of Kara's dream to become an "antamologist".

In 2011, Kara started her freshman year at Yale University, unsure whether she wanted to major in biology, archaeology, or geology. Her decision was solidified after taking her first entomology class. The professor, Dr. Marta Wells, had an infectious excitement about insects, exclaiming with glee each time a student found an insect, even for the most common species that she must have seen thousands of times before. The process of learning about insects in an academic setting confirmed her desire to pursue entomology as a career, so she applied to the Smithsonian Tropical Research Institute internship in Panama, where she worked the summer after sophomore year. She studied the natural history of a fascinating beetle that can change color from gold to metallic blue when disturbed. She loved her time there, but felt she wanted to find a research topic within entomology that might have a more immediate impact on people's lives.

She decided to try medical entomology. She joined the Yale Masters of Public Health 5-year joint program, which allows Yale undergraduate students to begin MPH classes during senior year and complete the program in a 5th year. Through the MPH program, she had the opportunity to research attractants for mosquito oviposition traps in Dominica and sugar lures in

Australia. These experiences confirmed for Kara that medical entomology was the right field for her and that mosquitoes were her study organism of choice.

Although Kara had found the topic that she wanted to study as a Ph.D. student, she first wanted to experience different ways of living outside of academia. The year after she graduated from the MPH, Kara worked as a wrangler on ranches in Wyoming and Argentina. These ranch jobs were very different from her goal of researching mosquitoes – but that is exactly why she wanted to experience them. She witnessed amazing beauty, learned a lot, and even found the best dog in the world, who she adopted and brought back to the US. The decision to take this year off reflects Kara's approach to life. She prioritizes balance and intentionally makes time to incorporate other interests into her life – from playing sports to hiking with her dog and spending time with her friends and family.

After the year of ranch life was over, Kara decided to join the Harrington lab. She was drawn to the lab by the mixture of research conducted there – from basic biology questions with implications for control and transmission to more applied questions with a direct and immediate impact on public health. As a Ph.D. student, Kara studied the feeding biology of an invasive species of mosquito, which you will read about in the coming chapters. The parts of her time as a graduate student that you will not read about here are the efforts that she made to advance science beyond the scope of her dissertation. Over the course of her Ph.D., Kara's philosophy as a scientist has evolved to prioritize the accessibility of science to the public and to policy makers. She believes that in order for science to meet its full potential, the public must understand and trust science, and legislators must be able to use the science to inform better policies. To this end, Kara has been involved in science communication and science policy on campus, in the community, and in the professional organizations of which she is a member. One of the efforts

that she is most proud of is founding an initiative called Vaccination Conversations with Scientists, which seeks to connect Cornell scientists with community members to answer questions about COVID-19 and vaccines.

One of Kara's goals during her Ph.D. was to conduct a research project on insecticide resistance in Iquitos, Peru. Unfortunately, the pandemic interfered with her plans, forcing her to evacuate back to the US soon after she had arrived there to start her project. She is very excited that her next step after her Ph.D. will be to return to Peru as a postdoctoral researcher to conduct a project similar to the one she had to put on hold in 2020!

Table of Contents

Table of Figures

Table of Tables

Introduction

During the adult life of a female mosquito, one of her primary needs is to find a host and take a blood meal, enabling egg production with the protein from the blood. Pathogen transmission is a devastating by-product of this blood feeding process, causing over 400,000 deaths per year. Although feeding is fundamental to both mosquito life history and pathogen transmission, there is much about this behavior that we do not understand.

Determining which host species each mosquito vector species utilizes is central to understanding blood feeding and pathogen transmission. The feeding patterns of a mosquito in nature dictate which reservoir species a mosquito will contact and, in turn, which viruses it may become infected with and ultimately transmit on to another host. Feeding pattern studies provide a snapshot of host usage at a certain time and place. However, they cannot be generalized to other settings because it is difficult to tease out the impact of environmental factors, such as host availability, and innate factors, such as host preferences. One method, albeit imperfect, to account for host availability is to assess host abundance and calculate feeding metrics, such as forage ratios and host availability indices. Feeding metrics provide more context to the observed feeding pattern, but leave many factors unknown, such as the impact of host defenses and the relative proximity or accessibility of hosts to the blood fed mosquitoes.

Another important piece to the puzzle is the contribution of host preferences to feeding patterns. Host preference can be measured directly though choice and no choice experiments. Determining the nature of the host preference of a mosquito species can help to isolate the impact of environmental factors on feeding patterns. However, host preferences can also vary between mosquito populations, so it is important to measure preference repeatedly across populations.

Combining together all these pieces of information regarding blood feeding biology informs a more generalizable interpretation of feeding patterns. With so many inputs influencing the final biting behavior, it is important to have layers of information regarding mosquito host preference, feeding patterns, and the environment that binds the two together.

However, even integrating across blood feeding patterns and host preference leaves a major gap in understanding mosquito feeding biology – namely because blood is not the only food source. Female mosquitoes also consume sugar from plants and other sources for energy. The interaction of blood and sugar feeding adds another layer of complexity. Sugar can increase mosquito survival, providing additional opportunities for blood feeding and increase energy stores for host seeking and biting persistence, but it can also decrease the proclivity to blood feed. As a result, it is also important to understand sugar feeding patterns and behavior. While not the proximate behavior enabling pathogen transmission, it is intimately connected to blood feeding biology.

Both blood and sugar feeding biology can also be harnessed for vector control through host odor and sugar-baited control techniques. For example, host odors are used as trap lures to improve trapping efficacy. Attractive toxic sugar baits use sugar to lure mosquitoes to consume the lethal treatment. As such, in addition to expanding our knowledge of fundamental aspects of mosquito life history and pathogen transmission, mosquito feeding biology research has the potential to improve our vector control tool set.

As a model organism to investigate feeding biology, my dissertation focuses on *Ae. albopictus*, a highly invasive mosquito species capable of transmitting over twenty pathogens. These pathogens have transmission cycles that involve a variety of hosts species. There have been limited assessments of *Ae. albopictus* host preference and sugar feeding. In contrast, there

have been numerous feeding pattern studies, but only two have been paired with host availability assessments. The feeding patterns studies have also been subject to misinterpretation, leading to conflicting narratives regarding *Ae. albopictus* feeding biology and highlighting the need for further research on the topic.

In this dissertation, the first chapter provides an in-depth discussion of feeding patterns and host preference, using *Ae. albopictus* as a case study. In chapter two, I described the feeding patterns of *Ae. albopictus* in residential areas and farms on Long Island and contextualized the patterns with two host availability measurements. This chapter also includes a comparison of *Ae. albopictus* fitness following feeding on blood from several different host species. In chapter three, *Ae. albopictus* host preference was measured with a dual-port olfactometer, comparing populations with high and low levels of human feeding. In the fourth and final chapter, I described the sugar feeding patterns of *Ae. albopictus* in Long Island, alongside an analysis of the contribution of several environmental and mosquito parameters. Together, the chapters of my dissertation seek to provide a holistic perspective of *Ae. albopictus* feeding biology, integrating across field and lab to better understand different pieces of this complex facet of mosquito biology.

Chapter 1 : Understanding and interpreting mosquito blood feeding studies: the case of *Aedes albopictus*[*]

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Abstract:

Blood feeding is a fundamental mosquito behavior with consequences for pathogen transmission and control. Feeding behavior can be studied through two lenses – patterns and preference. Feeding patterns are assessed via blood meal analyses, reflecting mosquito-host associations influenced by environmental and biological parameters. Bias can profoundly impact results, and we provide recommendations for mitigating these effects. We also outline design choices for host preference research, which can take many forms, and highlight their respective (dis)advantages for preference measurement. Finally, *Aedes albopictus* serves as a case study for how to apply these lessons to interpret data and understand feeding biology. We illustrate how assumptions and incomplete evidence can lead to inconsistent interpretations by reviewing *Ae. albopictus* feeding studies alongside prevalent narratives about perceived behavior.

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Highlights

Mosquito feeding behavior has profound impacts on disease transmission, vector control, and surveillance, necessitating unambiguous understanding of feeding patterns and preference.

Feeding patterns and preference are distinct concepts and should be evaluated independently through blood meal analyses and preference assays, respectively.

Potential sources of bias are often overlooked in feeding pattern studies and may have major consequences on conclusions. We identify these sources of bias and provide recommendations to reduce bias in study design.

Preference study design options are reviewed, alongside strengths and weaknesses of each.

A case study of *Aedes albopictus* blood feeding behavior demonstrates inconsistent interpretation of blood meal analyses in the literature, the need for more precise language when interpreting results, and a lack of preference studies for this species.

Diversity of Blood Feeding Behaviors

Nearly all mosquito species ingest nutrients from vertebrate blood to produce eggs, making their drive to blood feed exceptionally strong. During feeding, female mosquitoes can release pathogens into the host or become infected themselves. For this reason, understanding blood feeding behavior has been a long-standing priority for entomologists seeking to interrupt mosquito-human contact, thereby reducing pathogen transmission. Despite intense focus, we do not fully understand mosquito feeding behavior, including host choice variation and factors

influencing mosquito feeding patterns. Sugar feeding often supplements blood nutrients for females and is the only nutrient source for males – yet it can be overlooked despite its impact on modulating blood meal intake and digestion rates [1].

Mosquito blood feeding behavior can be complex; some species feed on a wide range of hosts, from humans to frogs, and express varying degrees of specificity for those animals [2]. Specialist mosquitoes prefer to feed on a particular host species or animal class and tend to do so at a high rate. For example, the dengue vector, *Aedes aegypti aegypti*, prefers humans to other animals in choice experiments [3], and field assessments of feeding patterns demonstrate frequent human feeding [4]. Mosquitoes with such pronounced preference for humans are considered **anthropophilic** (see **Glossary**) and those that feed often on humans are called **anthropophagic**. Other mosquitoes have broader host preferences for numerous species within a class. For example, the West Nile virus (WNV) vector, *Culex pipiens pipiens*, typically prefers American Robins (*Turdus migratorius*) compared to other birds [5], but generally prefers birds to mammals [6]. Mosquito species that prefer birds are considered **ornithophilic,** whereas those preferring mammals are **mammalophilic.** Mosquito species that are not specialists are considered **generalists**, feeding on a diverse array of animals, and likely feeding on the first available host encountered. The term generalist can be employed for species that display a host class level preference but are generalists at the species level or for those that have no preference across class and species levels. **Opportunistic** feeders may have a host preference but will readily feed on a diverse array of animals. Often, terminology and evidence for classifying mosquito vectors is confusing; thresholds for classification are unclear across the spectrum from generalist to specialist feeding types (Box 1).

Here, we review the terms and biology associated with mosquito feeding patterns and preferences. To aid future studies, we provide guidance on sampling approaches and criteria for classifying mosquitoes into blood host associated groups. We then present a case study examining current knowledge about feeding patterns and behavior of the globally invasive vector, *Aedes albopictus.*

Box 1. Recommendations for feeding behavior study design and interpretation.

1. *Report results accurately:* Ensure that conclusions are based on the available data -it is not possible to definitively evaluate preference with blood meal data, so do not attempt to infer preference from field-collected blood meals. Instead, consider addressing the local ecology and pathogen transmission risk revealed by the observed feeding patterns.
2. *Expect variation:* Demonstration of pattern or preference at one point in time or place usually is not generalizable to other locations and times. Variability exists among populations, so it is important to address these research questions repeatedly over time and space.
3. *Account for host availability:* When performing blood meal studies, account for host availability. The more detailed the record of host availability, the better, but even a brief survey can provide valuable context.
4. *Choose the appropriate preference assay format:* Based on available resources and equipment as well as the current knowledge for the species in question, choose the most informative preference assay format possible. It may be helpful to move progressively from smaller scale, more controlled assays to larger scale, more natural formats.
5. *Reduce and report sources of bias:* When conducting blood meal studies, reduce sources of bias outlined in Table 1.1, and when not possible, clearly report the biases that exist.
6. *Observe guidelines for terminology use*: Unfortunately, the blood feeding literature does not contain recommendations for how and when to use terminology. We suggest the following

guidelines based on a theoretical thought process (please see the full explanation in supplementary Box S2). These suggestions are meant to provide a framework to improve consistency in terminology use within the field. Future research should seek to refine these suggestions through modeling efforts.

a. -philic: At least three independent, high quality preference studies have shown preference for a given animal species or class compared with a diverse array of other species/classes. Mosquito species must be at least two times more likely to choose a given species/class compared with alternatives; simple statistical significance is not sufficient – it must be biologically significant.
b. -phagic: At least 33.3% of field-collected blood fed mosquitoes contain blood of a given host species/class in at least 3 studies, regardless of setting.
c. When a minimum of three independent studies have not replicated a finding, be cautious with terminology and use qualifying statements such as "indicative of" a certain behavior.

Distinction between host preference and feeding patterns

The difference between **feeding patterns** and **host preference** is a key distinction when discussing feeding behavior [7]. Feeding patterns describe the hosts which mosquitoes feed on in nature and can be influenced by myriad factors both intrinsic and extrinsic to the mosquito species, including host availability to mosquitoes, host defenses, and mosquito host preference [8]. In contrast, host preference is a solely intrinsic trait describing mosquito species' tendency to select certain hosts over others. Preference is determined genetically and can evolve with fitness benefits after feeding on a particular host species [8, 9]. Different experimental techniques are recommended to assess patterns and preference, as described below.

Measuring Feeding Patterns

Determining host feeding patterns is useful to understand feeding frequencies on hosts present in a specific place and time. Although feeding patterns do not necessarily reflect preferences [7], they can be useful for understanding context-dependent biting risk [10]. When many robust feeding patterns studies are conducted over time, resulting trends can suggest preference. To assess feeding patterns, researchers typically collect blood-engorged mosquitoes from the environment and determine their host blood source [7]. Feeding pattern studies based on blood meal analysis can be biased in several ways, leading to a predominant host that does not necessarily reflect innate mosquito preference for that host. Whenever possible, it is important to consider bias when conducting these studies. Sources of bias can arise from both field sampling methods [11-14] and blood meal determination [15-17] and can reduce the concordance between patterns and preference. Recommendations for overcoming bias are presented in Table 1.1. Some bias reduction techniques require additional time and effort compared with alternatives, e.g. active collections are laborious in comparison to host-seeking traps, and a combination of indoor and outdoor collections is often less efficient per unit collection time than indoor collections alone, especially for primarily **endophagic** species (e.g. *Aedes aegypti [18]*). However, the quality and utility of data can be improved with these bias reduction methods.

TABLE 1.1: BIASES IN BLOOD MEAL STUDIES AND RECOMMENDATIONS FOR REDUCING BIASES

Source of Bias	Influence on Results	Recommendations for Reducing Bias
Site location		
General	Collection site determines availability of host species. If the preferred host is unavailable or rare at one site and common at another, observed feeding patterns will be different [11].	Include sites where the mosquito is abundant. If examining transmission risk, include sites where the disease is endemic or there is concern for introduction. Ideally, include sites with variable habitats and host availability (e.g. **peridomestic**, **non-peridomestic**) [78]. This is not necessary if the goal is simply to assess human exposure in urban environments.
Indoor/Outdoor	For some species that enter houses, and in areas where houses are susceptible to mosquito entry and egress, sampling indoors versus outdoors can impact level of anthropophagy detected [12, 13, 112].	Sample both indoors and outdoors, especially in locations with open housing where mosquitoes can easily fly in and out. Outdoor sampling typically yields lower numbers of blood feds per unit sampling time for some species – account for this in sampling strategy.
Host seeking traps		
Partial meals	Collection of host-seeking mosquitoes with specialized traps may bias towards defensive hosts if defensive actions result in partial blood meals, leading to seeking a second meal [14]. Although this effect has rarely been reported, it should be considered.	Additional evidence is necessary to determine presence/ extent of bias across species and corresponding need to reduce bias. Use resting boxes [14] or active collection methods such as aspiration of resting replete females [87].
Odor lure	Collecting mosquitoes attracted to specific host odors (e.g. BG trap lure) may bias interpretation of results towards that host, potentially obscuring individual attraction variation within the	If there is a risk for this bias, use active collection methods, traps without specific odor blends, or resting boxes.

	population. This may be especially important for species with high levels of genetic variation for preference [9, 48].	
Blood meal ID		
Serological methods	Serological methods (e.g. ELISA) require *a priori* selection of host-specific antibodies or antisera and exclude all other hosts [4]. Some antibodies lack species level specificity, resulting in binding to non-target species sera [113].	Use PCR of species-variable regions (Cyt B, COI) followed by Sanger sequencing [113] or Illumina sequencing [15] rather than serology when possible. If the main goal is to understand feeding patterns on a single host at risk, such as humans, serological methods can still be useful.
PCR	Some primers are designed to only amplify sequences from certain groups of animals or achieve superior amplification for particular species, resulting in either purposeful or accidental exclusion or suppression of other hosts [15-17]. Contamination of samples can result in misidentification.	Test primers on control DNA from all possible hosts in collection area or use primers that have been thoroughly tested on a diverse range of animals. If targeting sequences for one class of animal, use or design additional primers to amplify other animal classes. Ensure sterility of the laboratory environment during analysis.
Mixed meals	It is difficult to sequence mixed meals when utilizing PCR [113] followed by Sanger sequencing, which may reduce identification of certain hosts frequently found in mixed meals (e.g. defensive hosts increase partial meals, leading to a higher tendency to take a second meal).	Clone PCR product and sequence each unique clone [113] or use Illumina sequencing [15].
Pre-blood meal scenario		
Host defenses	Mosquitoes may be diverted or killed by host defenses [114]. Although mosquitoes were initially attracted to the host, this will not be reflected in blood meal collections.	This is not possible to resolve in field blood meal collections, but separate assessments of host defenses can provide additional context. Unfortunately, we know little

		about the impact of defensive hosts on mosquito blood feeding.
Host availability	Host availability at the time of host seeking is unknown, so it is not possible to conclude whether a choice was made between animals.	Frequent host census can be performed to calculate feeding metrics (e.g. host feeding indices, forage ratios) [19, 20] close to the time of mosquito collections. These metrics provide additional context but do not resolve availability at the exact time/place of feeding.
Resting location	Mosquito resting location relative to host location (e.g. for host roosting, foraging, working) may influence the probability of contact along mosquito host-seeking flight paths [115].	Mosquito sampling should address spatial distribution of resting and host seeking mosquitoes. Animal census should note location of animals within sites. Together, these data can inform the role of flight paths on host contact. Especially important for species strongly impacted by landscape features and with predictable flight paths.
Sampling		
Size	A small sample size will likely bias conclusions about feeding patterns towards the most common hosts.	Enhance sampling efforts to achieve larger sample size. Collecting engorged mosquitoes can be difficult and time consuming. Unfortunately, currently available collection techniques yield low numbers of blood fed mosquitoes. Expand collection efforts over time and space to increase sample size.
Season	Feeding patterns can vary across a season, so a narrow sampling time may not provide a full picture of feeding behavior [116, 117]. Shifts in host feeding patterns can be important for understanding transmission risk [118].	Sample across the entirety of the active season when possible. This is essential for species with avian hosts that nest and migrate at different times of the year.

Despite the distinction between patterns and preference, many researchers claim preference when a certain animal is fed on more often than others. Although this interpretation is tempting, it can be misleading and should be avoided. Blood meal analysis does provide valuable information about actual contact that mosquitoes have with hosts in nature, which ultimately influences pathogen transmission. It is important to determine whether mosquitoes feed, even infrequently, on non-preferred hosts that may be pathogen reservoirs or may dilute transmission of host-specific pathogens. Even a strong preference does not guarantee exclusive feeding. Blood meal analysis provides key information to this end.

The results of blood meal studies can be highly season- and location-specific, necessitating localized studies. However, results can become more generalizable by contextualizing host availability patterns via host feeding indices (HFIs) or forage ratios (FRs) informed by a host census [19, 20]. HFIs compare the relative frequency of successful feeding on two animals by dividing the proportion of blood meals taken from each animal by the proportion of individuals of each species per geographic unit and is calculated as follows [19]:

$$\mathrm{HFI} = \frac{\mathrm{B}_x/\mathrm{B}_y}{\mathrm{H}_x/\mathrm{H}_y}$$

where B_x and B_y represent the average number of blood meals from host *x* and host *y* per geographic unit and H_x and H_y represent the average number of host *x* and host *y* present per geographic unit. HFIs can be based on abundance alone or time-weighted [21]. FRs theoretically compare relative propensity for successful blood feeding on all hosts present by dividing the proportion of all blood meals taken from an animal by the proportion that the animal comprises of the full host population. It is calculated as follows [20]:

$$\frac{\text{Number of blood meals from host } x \text{ / Total number of all blood meals}}{\text{Number of host } x \text{ in the population /Total number of all hosts in population}}$$

Host census for these calculations take various forms, including household interviews of human and pet abundance and/or time outside, camera traps, transect surveys, and external presence or abundance data [21-24]. None of these methods are perfect, with forms of bias and error in each. Furthermore, a general knowledge of availability does not resolve other unknown factors, such as diversion or host defense induced mortality and availability of hosts at the exact time of host seeking. Therefore, HFI and FR values suggest host preference or avoidance but cannot prove it. They can, however, make blood meal analyses more generalizable by providing additional host context, enabling more robust conclusions about host associations and transmission risk.

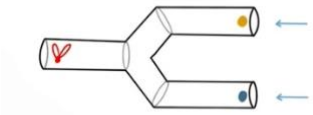
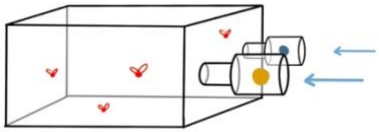
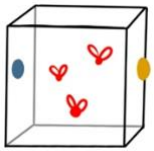
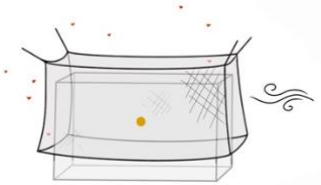
Researchers in Gabon used an indirect method to overcome the persistent challenge of limited sample size in blood feeding studies [25]. *Anopheles* feeding patterns were inferred by detecting host specific blood parasites in non-engorged mosquitoes, increasing the sample size ten-fold compared with blood meal analysis [25]. Future studies could explore similar indirect methods to understand feeding patterns.

Host Preference

Host preference can be directly assessed via choice or no choice experiments, which take many forms, each with their own set of advantages and disadvantages (Table 1.2). **Choice experiments** require mosquitoes to choose between two hosts, allowing direct assessment of relative attraction [9, 26, 27]. They are useful for estimating risk when both hosts are available. These experiments assess **host choice** at the individual level, but aggregation of individual responses enables conclusions about host preference, which is determined by the frequency at which mosquitoes make a certain choice. Some experimenters conduct **no choice experiments** to determine general tendencies of mosquitoes to be attracted to a host in the absence of other

46 available animals [26, 27]. Both instances may arise in nature. However, no choice experiments
47 have limited utility when conducted at small scales and with starved mosquitoes, which may tend
48 to feed indiscriminately under such circumstances. In addition to assessing degree of host
49 attraction, both choice and no choice preference experiments are an important tool to investigate
50 potential molecular and physiological underpinnings of feeding behavior [2, 3].

51
52 **TABLE 1.2: ASSAY CHOICES FOR HOST PREFERENCE ASSESSMENT**

Method and Example References	Advantages	Disadvantages
Apparatus		
* a)  b)  c)  d)  e) 		
a) Y-tube [26, 46, 49]	Air movement	Small scale; potential for observer odor bias
b) Wind Tunnel [34, 40, 44, 50]	Air movement, realistic odor plume, allows for detailed analysis of host-seeking flight behavior	Specialized equipment, time-consuming; potential for observer odor bias; often assesses a single attractant
c) Dual-port Olfactometer [3, 45]	Air movement; does not require behavior monitoring	Requires port entry
d) Choice Chamber [3, 40]	Limited need for specialized equipment	Small scale; often does not include air movement which makes cues for mosquito orientation less clear; potential for observer odor bias
e) Baited traps [5, 31, 33, 38, 50, 90, 119]	Can be used in field or semi-field settings with wild or colonized mosquitoes; does not require behavior monitoring	Some trap designs require port entry; aspects of trap design may influence attraction (e.g. visual cues)
Location		
Laboratory [33, 49, 50]	High degree of control of external variables	Unnatural setting; small scale; often uses long term laboratory-colonized mosquitoes that may behave differently than field mosquitoes

Semi-field [5, 31]	Some variables are reflective of natural setting while others remain controlled; medium scale	Setting is not fully realistic; uncontrolled variables can make interpretation challenging; often uses long-term laboratory-colonized mosquitoes that may behave differently
Field [31, 38, 50, 90]	Natural setting; natural (large) spatial scale; naturally occurring, field-derived mosquitoes	Less control of external variables
Odor Source		
Live animal [5, 26, 38, 50]	Ensures complete odor profile is displayed at correct levels; odor is paired with additional realistic cues (heat, visuals) for studies of holistic host seeking behavior	Requires animal-use permits and logistics of animal care; may exclude certain animals due to size or permitting constraints; depending on presentation format, difficult to control for host defenses; challenging to disentangle other cues (heat, visuals) from odor cues
Natural scent [31, 49]	Inexpensive; relatively easy to collect and present in an assay	Variable odor dissipation rates from collection material; odor collection material will be in close contact with a particular part of host body, not necessarily reflective of full body odor; strength of odor will be relatively weak compared with a live animal; requires animal-use permits; odor is not necessarily paired with other cues (heat, visuals), providing incomplete attraction profile
Synthetic scent [31, 33]	Allows for identification of particularly attractive components of host odor; scalable for surveillance and control	Relatively expensive collection process requiring specialized equipment; odor is not a complete blend emitted by natural host, which may or may not be paired with other cues (heat, visuals), providing incomplete attraction profile
Behavior		

Biting/Probing [38, 50, 120]	Most proximate to the end point of interest – host feeding	Biting may require use of live animals and exposes animals to infection if using wild mosquitoes; difficult to eliminate impact of host defenses; probing (e.g. through a mesh barrier or artificial feeding membrane) requires realistic presentation of cues (e.g. heat) to prompt feeding behavior off-host
Landing [40, 44]	Simple experimental set up	May over-estimate preference because mosquitoes may depart host after landing before feeding in nature; may under-estimate preference if presentation of cues (e.g. heat) is not realistic and mosquitoes are not prompted to land; if conducted with live hosts, exposes them to bites and potentially to infection
Port Entry [5, 31, 33, 50]	Simple experimental set up; end point can be evaluated without constant observation	Some port entries elicit avoidance maneuvers by mosquitoes
Directional flight [26, 49]	Does not require host cue presentation in a natural format that would elicit downstream behaviors (landing, biting)	A mosquito that flies towards a cue will not necessarily feed on the source; requires observation

* yellow and blue circles represent host odor source; red mosquito sizes are roughly reversely proportional to size of apparatus; blue arrows represent forced air flow; black lines represent natural air flow. Note that many variations of these apparatuses exist and the drawings are general examples, not intended to represent all possible designs. This is particularly true of baited traps, which include many vastly different designs.

Preference experiments can be conducted with live hosts, host-derived odors or synthetic blends. Live hosts ensure that all components of the odor are displayed at natural levels [28] in conjunction with other cues, such as potential visual cues, heat, and CO_2. However, it requires appropriate animal handling permits and can be logistically difficult. Host-derived odors can be acquired through methods including prolonged contact with material (e.g., glass beads or fabric) followed by presentation as an odor source or collection of headspace volatiles [29-31]. However, volatile compounds have different dissipation rates, so collected odor profiles change over time [32]. Synthetic blends allow identification of host odor components that are optimally attractive to mosquitoes. This is an essential step towards creating odor baits for operational control and surveillance [33]. Presentation of host-derived and synthetic odors can be paired with other cues, such as heat and CO_2, to enhance attraction [34, 35]. When visual cues are incorporated, they are typically simplified to a dark visual contrast [34, 36, 37], which may not be a realistic representation of visual host cues. Depending on the experimental purpose, isolated odor cues may provide incomplete assessments of attraction.

Variation in behavioral response assessed in preference experiments is another consideration. Some experiments assess biting or probing [38, 39], whereas others assess landing, upwind flight, or portal entrance [26, 29, 40]. Measurement of biting or probing behavior is the most relevant endpoint, but experimental and ethical limitations may preclude use of this response. When biting is measured with unrestrained hosts, host defenses may impact results. These assessments also may be conducted under different conditions, including laboratory, semi-field, and field [30, 31]. Laboratory experiments provide the highest degree of control, whereas field experiments reflect natural conditions. Laboratory and semi-field experiments often use laboratory-adapted, colonized mosquitoes which are known to have

limited trait diversity and altered genotypes [41, 42]. To avoid potential trait evolution through laboratory adaptation, field-derived or low generation colonized mosquitoes should be used whenever possible. When colonization is necessary, selection based on host preference should be avoided by ensuring high feeding rates [43]. High feeding rates are more likely to retain a diversity of host preference genotypes rather than selecting for those individuals that feed most readily on a given host blood source in the lab. Field experiments utilize naturally occurring, genetically diverse mosquitoes.

Preference assays can be conducted with different experimental tools. The wind tunnel is a common tool which tests upwind flight response of mosquitoes towards host stimuli. The movement of carbon-filtered air with odor generates a realistic plume, allowing detailed analysis of host-seeking behavior [34, 44]. Wind tunnels are frequently used with a single stimulus, reflecting a no choice attraction scenario; relative attraction levels can be compared among stimuli to reveal aspects of preference. Dual-port olfactometers are similar devices; however, they are designed for simultaneous presentation of two treatment/control odor sources, are typically smaller, and require mosquito entry through a port [3, 45]. Choice chambers allow presentation of two hosts/odors with less specialized equipment, but may lead to less realistic orientation to odors due to small size and lack of air movement [3, 40]. Another method, the Y-tube olfactometer, has one arm for entry and two for odor/control presentation. It often includes movement of carbon-filtered air to the entry portal [46], but is smaller than the afore-mentioned methods, leading to less realistic mosquito flight and orientation. Baited traps may be used to assess relative attraction to certain odor compounds or hosts [5, 31]. These experiments are typically conducted at larger scales (semi-field or field); however, behavioral response often requires port entry into the trap, potentially reducing capture and limiting correspondence with

attraction [47]. Baited traps are most representative of how innate preferences will translate to surveillance and control efficacy. Bias can affect the above experimental designs through odor residues left on equipment while handling [46] or through observer presence. These can be minimized with use of cameras in lieu of human observers [40], carbon-filtered air [44], proper cleaning procedures [46], and wearing gloves while handling equipment [45].

Variability in feeding preferences

We have discussed ways in which observed feeding patterns can vary as the result of extrinsic factors, such as host availability. Additionally, variability in innate preference may influence observed differences in patterns. Feeding preferences may vary geographically and temporally [43]. Importantly, detection of feeding preference in one population at a particular time point does not mean that the same preferences will be detected in other populations or the same population at another point in time. Preference is often determined genetically and is therefore subject to evolution [8, 9, 48]. It can vary between individuals and populations and may evolve in response to control measures.

Preference variation between individuals

At the smallest scale, host preference may vary between individuals, the unit upon which evolutionary forces act, leading to the establishment of species level preferences. For the relatively zoophilic malaria vector in Tanzania, *Anopheles arabiensis*, a chromosome inversion significantly predicted whether individuals fed on cattle or humans [48]. Experiential learning is under-studied, but could introduce variation in individual host preferences; host defenses can deter mosquitoes from seeking that host species thereafter [49].

Preference variation between populations

Natural selection can act at the local level to create populations with different host preferences. Intraspecific variation was reported for *Culex annulirostris*, with two geographically isolated South Australia populations expressing different host preferences – one for guinea pigs and the other for chickens [50]. Even amongst species that are classically considered highly anthropophilic, such as *An. gambiae*, intraspecific variation in host preference exists among populations [26]. In certain cases, these population-level differences become fixed traits of diverging sub-species, as in the cases of *Cx. pipiens molestus* and *Ae. aegypti aegypti* [3, 6]. Below-ground *Cx. pipiens molestus* diverged from above-ground *Cx. pipiens pipiens;* landing assays demonstrated that *Cx. p. molestus* from Chicago fed preferentially on humans compared to chickens whereas *Cx. p. pipiens* fed preferentially on chickens [6]. The domesticated sub-species, *Ae. a. aegypti* diverged from the forest-dwelling *Ae. a. formosus*; preference assays demonstrate genetically determined human preference in *Ae. a. aegypti* for humans compared to guinea pigs, whereas *Ae. a. formosus* preferred guinea pigs [3]. In sub-Saharan Africa, the ancestral range of *Ae. aegypti*, human preference variation has been linked to local human density and dry season intensity [43].

Preference variation in response to control

In addition to natural phenomena, host preference can evolve in response to vector control [51]. Mosquito populations have repeatedly shifted host use after implementation of control measures, such as insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) [52-54], though

not for all locations, species, and treatments [53, 55]. The degree to which these shifts were the result of behavioral plasticity interacting with reduced human availability or evolution in response to selective pressure is unclear [8]. An evolutionary model suggests that this change is possible through natural selection, resulting in increased zoophily, however, definitive evidence of such evolution is lacking [56]. In contrast, in Burkina Faso, ITN coverage appeared to interact with *An. gambiae* feeding plasticity, resulting in large proportions of non-human blood meals, but preference was unchanged; the population retained strong anthropophilic behavior when presented a choice [57].

Impact of Host Preference and Patterns on Epidemiology

Host preference and patterns can impact several aspects of mosquito surveillance and control (Box 2), as well as disease transmission ecology and epidemiology. Accurate assessment and interpretation of feeding patterns and preference data are essential, as mosquito feeding behavior is pivotal in determining and predicting disease dynamics. Blood feeding patterns (including species and breadth of hosts) impact probability of acquiring and transmitting pathogens (Figure 1.1). Generalist feeders may have lower probability of transmitting a single host pathogen compared with specialist feeders due to decreased likelihood of imbibing two sequential blood meals from the same host species. In contrast, generalist feeders may be more likely than specialists to serve as bridge vectors for zoonotic infections by transmitting pathogens between a reservoir host and other susceptible host species.

Box 2. Impact of Host Preference on Vector Surveillance and Control

Many tools available for mosquito surveillance and control depend on host seeking biology, reinforcing the importance of accurately understanding host preference. Improved understanding of preference can enhance efficacy of control tools and refine accuracy of surveillance.

One of the most classic malaria control tools, long-lasting insecticidal nets (LLINs), exploit host seeking behavior by using protected humans as bait to lure mosquitoes and induce mortality through contact with the net. The success of LLINs in reducing mosquito populations is therefore more successful for more anthropophilic and endophagic species [98]. Other control techniques target less anthropophilic species. **Zooprophylaxis** is used in some situations to draw host seeking mosquitoes away from humans to non-reservoir animals, thereby reducing opportunities for transmission. A review of this method for malaria control suggests that the method works best on **zoophilic** and **exophagic** vectors [99]. Attraction to non-human animals can be combined with insecticide treatment of animals to induce mosquito mortality [100].

Host preference is also utilized in developing attractants derived from host odor for host-seeking traps, some of which are made to specifically emulate a human host while others are more general [101, 102]. These semiochemicals attract different mosquito species with varying efficacy [103-106], so adapting trap attractants for the target species improves results.

Host-seeking traps are often used for surveillance, so differences in trap efficiency due to differences in host-seeking biology between species can lead to differences in perceived abundance and public health importance by species [107-109]. Because trapping often informs implementation of control, lower trap attraction for some species may yield poor data for informing control decisions.

Despite the potential of using specific odors in host-seeking surveillance traps, the technique is under-utilized [110]. Discovery and testing of additional host semiochemicals is needed. Importantly, an improved understanding of the host seeking biology for target mosquito species will optimize attractant composition. Due to the multimodal nature of host seeking behavior, host semiochemicals may need to be

paired with other attractants, such as carbon dioxide, heat, or visual cues for optimal attraction [35-37, 111].

Finally, continued investigations of host preferences and feeding patterns are essential to identify and understand amplifying and bridge vector species and correctly target control efforts.

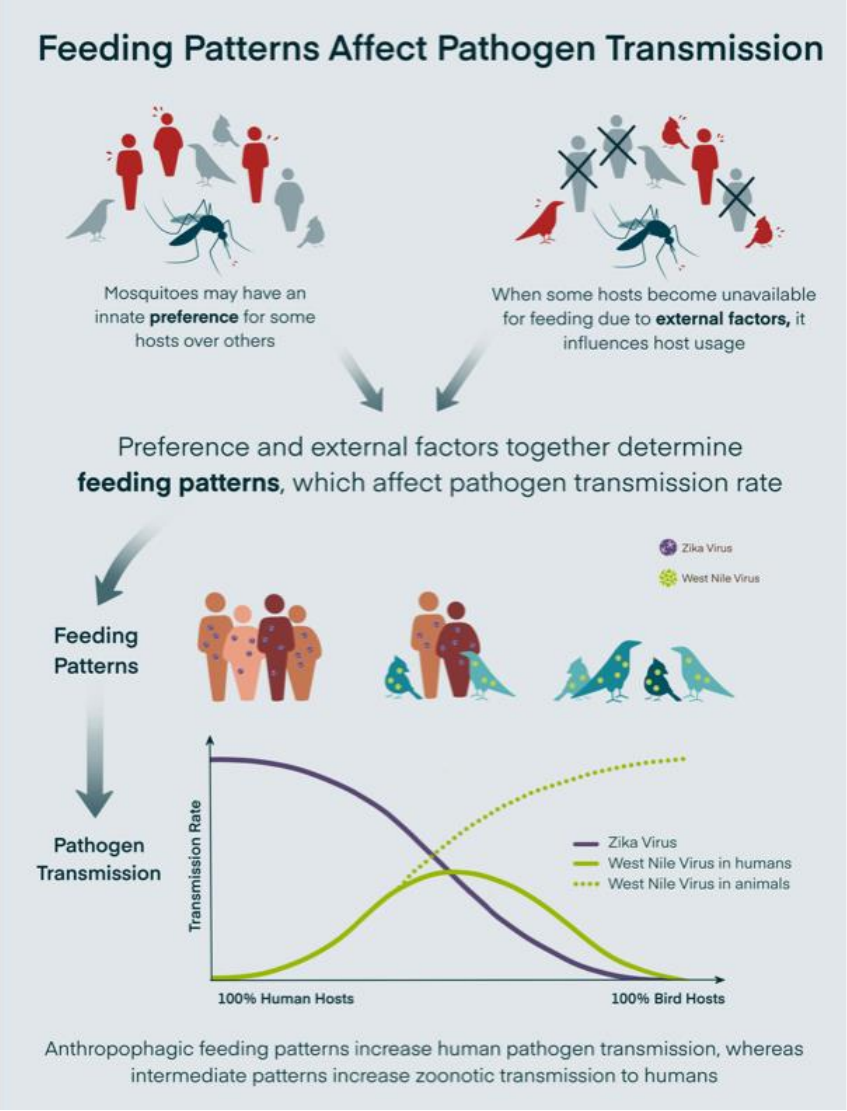


FIGURE 1.1: FEEDING PATTERNS AFFECT PATHOGEN TRANSMISSION

Mosquitoes may have an innate preference for particular host species over others and therefore tend to feed on that species when available (feeding indicated by red coloration of hosts in top panel). When that species is rare or unavailable due to external factors, host usage can shift to include more readily available hosts. Innate preference and external factors together determine feeding patterns, which in turn influence pathogen

transmission. Mosquito transmission of anthroponoses (e.g. Zika virus, represented by green circles in hosts and green line on chart) is most efficient with highly anthropophagic feeding patterns. Mosquitoes can serve as optimal bridge vectors of zoonoses (e.g. West Nile Virus, represented by blue circles in hosts and solid blue line on chart) to humans when feeding patterns include moderate levels of both anthropophagy and zoophagy. Enzootic transmission of zoonoses (blue dotted line) among animal reservoirs is maximized with high levels of zoophagy. Transmission rate trends for Zika and WNV are theoretical and do not reflect real transmission data. Illustrator: Sage McKeand

The impact of host feeding patterns on pathogen transmission can be assessed conceptually with the vectorial capacity equation, which, in its most basic form, utilizes five variables to predict the average number of new infections originating from one infectious vector-borne disease case per unit time:

$$VC = \frac{ma^2bp^n}{-log_e p}$$

where *m*= number of mosquitoes per host, *a*= daily blood feeding rate, b= transmission rate among exposed female mosquitoes, *p*= probability of daily survival, and *n* = extrinsic incubation period [58-60]. The exponentiation of daily feeding rate, reflecting the necessity of feeding twice to become infected and transmit a pathogen, makes mosquito feeding behavior the most impactful variable in the equation after survival. For example, vectorial capacity based on experimental vector competence data and hypothetical host preference indices supports the notion that *Ae. albopictus* has lower capacity for dengue virus than *Ae. aegypti*, especially when the host preference index is considered lower based on reported feeding patterns in the literature [61].

The importance of host preference is borne out in epidemiological models that incorporate feeding patterns to predict disease dynamics. A basic reproductive number (R_0)

model indicated that exceptionally low rates of human feeding by *Ae. aegypti* in Texas was responsible for low levels of local Zika virus transmission [62]. In the WNV multi-host disease system, an empirically informed transmission model that included *Cx. pipiens* feeding indices accurately predicted WNV infection in three of four sites; subsequent sensitivity analyses showed that feeding index was the most influential parameter in determining severity and timing of WNV infection peak [63]. The same model using *Cx. quinquefasciatus* experimental host choice data indicated that generalist blood feeding on less competent hosts led to similar WNV transmission potential as aggregated blood feeding on highly competent hosts [64].

Considering the evidence for several mosquito vectors showing that inter- and intra-species blood feeding variation can lead to different disease transmission outcomes, accurate interpretation of mosquito feeding preferences and patterns is essential to predict and mitigate disease spread.

Case study: *Aedes albopictus*

Aedes albopictus provides a useful example to understand interpretation of blood feeding studies. It is a globally invasive species, demonstrating vector competence for over 20 pathogens with varying transmission cycles that utilize numerous host species [65]. Understanding *Ae. albopictus* feeding behavior is essential to assess public health risk for the range of its associated pathogens. However, there is little consensus in the literature regarding its host preferences and feeding behavior [66, 67]. Some researchers consider *Ae. albopictus* anthropophilic [65, 68, 69], whereas others consider it opportunistic [61, 67, 69, 70] or generalist [71-73]. Lack of consensus is due to variability in methodology, uncertainty surrounding feeding behavior categorization, and lack of controlled assessments of feeding preferences.

We reviewed 38 studies of field-collected *Ae. albopictus* blood meals (supplementary Table 1.3; search strategy in supplementary Box S1). However, only 19 had sample sizes of over 60 blood fed mosquitoes (results in supplementary Table 1.4); below, discussion of *Ae. albopictus* feeding patterns is limited to this subset [4, 21, 68, 70, 74-87].

Across the 19 studies, the average percent of human blood meals detected in *Ae. albopictus* was 51.8% ± 32.7 sd (range: 3.9 – 100%). Number of different host species detected in blood meals ranged from 3 to 15, although some studies may have underestimated diversity due to use of serological tests (Figure 1.2). Humans were the most common blood source in 14 studies, rabbits in two studies, and dogs, cats and rats in one study each. Eleven reported bird blood meals, with implications for transmission of numerous arboviruses for which *Ae. albopictus* is a competent vector.

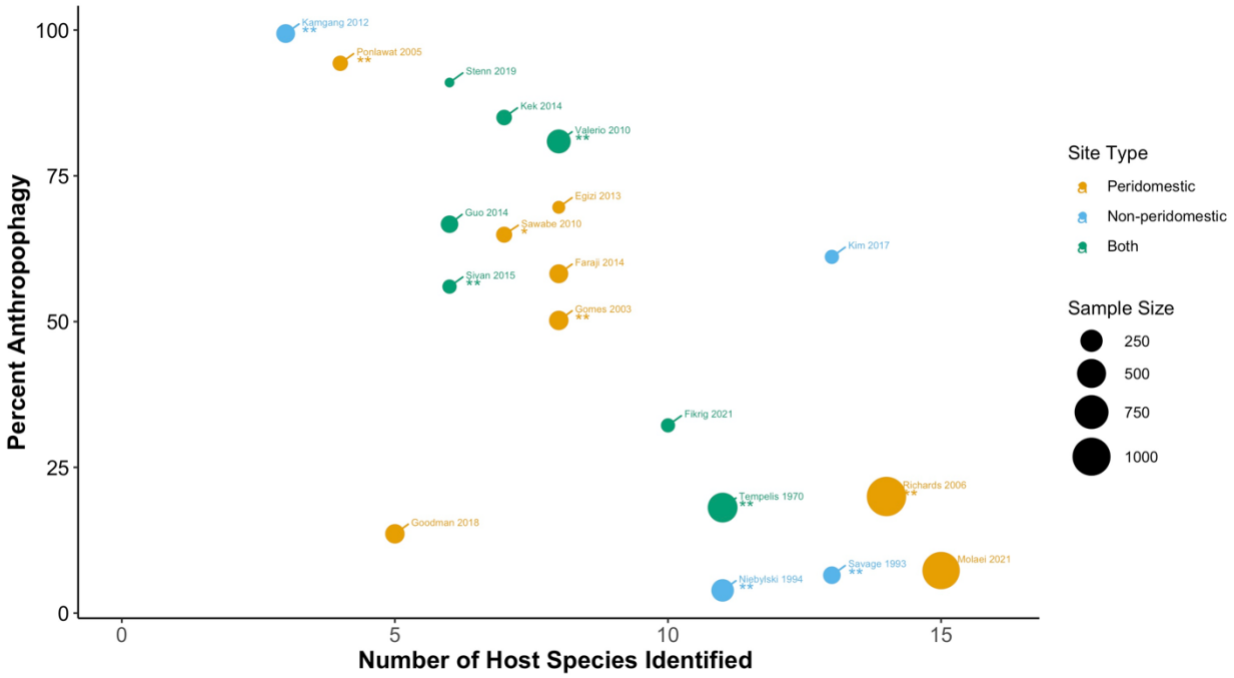


FIGURE 1.2: *AEDES ALBOPICTUS* FEEDING PATTERNS.

Percent total human blood meals (anthropophagy) and number of host species identified (host diversity) are displayed for each of the 19 *Ae. albopictus* blood meal analysis studies. Point size reflects relative sample size and

color indicates whether collections were conducted at peridomestic, non-peridomestic, or both site types. ** indicates use of serological methods and * indicates PCR-identified samples (identification to class and not species for at least one sample).

Across the 19 studies, bird blood meals were detected in an average of 4.5% ± 7.2 (range: 0 – 25.6%) of engorged mosquitoes. Seven studies detected reptile blood from *Ae. albopictus* and three detected amphibian blood, although the maximum percentage of blood meals derived from these sources was low (3.9% and 2.2%, respectively). In all cases, at least 72.2% of blood meals were mammalian, and in five studies, all blood meals were mammalian. *Aedes albopictus* is a vector of dog heartworm (*Dirofilaria immitis*), which involves several **definitive hosts**, including dogs and wild canids. Dog blood was detected in 14 studies (mean= 5.9% ± 6.0, range= 0 – 19.8%) and fox blood in one study (0.2%). Cats, which can become ill but are dead-end hosts for *D. immitis*, were frequent *Ae. albopictus* hosts (detected in 14 studies, mean= 10.5% ± 13.2, range= 0 – 50.5%).

The cause of variability in host feeding patterns among studies was unclear, but one factor may be host availability. Quantitative host availability data were collected in only three studies, with differing results [21, 76, 87]. In North Carolina, HFIs based on host abundance showed that cats and dogs were over-utilized compared with humans, but authors concluded the opposite when accounting for time hosts spent outside [21]. In contrast, New York *Ae. albopictus* over-utilized cats and dogs compared with humans based on both abundance and time-weighted feeding indices [87]. Forage ratio calculations in that study showed disproportionately high rates of feeding on cats and opossums and disproportionately low rates on raccoons, squirrels and birds. In Brazil, feeding indices based on animal abundance indicated

that *Ae. albopictus* over-utilized humans compared with chickens and dogs and, to a lesser extent, cattle and horses [76].

Several researchers have reported the impact of host availability through comparisons of peridomestic and non-peridomestic sites, consistently demonstrating that human blood meal proportion decreases, and host diversity increases in non-peridomestic settings where humans are typically less abundant [78, 82, 83, 87]. We might expect that studies focused solely on peridomestic or non-peridomestic sites would follow a similar pattern, however, this is not the case. In Cameroon, collections were performed in non-peridomestic locations (outdoor recreation/equestrian centers), but 99.4% of engorged mosquitoes contained human blood despite availability of many other hosts [79]. In contrast, collections in North Carolina and Virginia were conducted on residential properties but had relatively low levels of human feeding (20.0% and 7.3% respectively) and high levels of diversity (14 and 15 hosts respectively) [21, 86]. Additionally, in urban Baltimore, Maryland, *Ae. albopictus* fed infrequently on humans (13.6%) but exhibited low host diversity (five hosts) [85].

Among the 16 studies that did not quantitatively assess host availability, authors of 13 inferred preference from feeding pattern results. Terms and phrases used include: "anthropophilic", "generalist", "host preference", and "prefers to feed". These statements of preference based on feeding patterns were then referenced by other authors regarding *Ae. albopictus* biology (e.g., [88, 89]), creating an unsubstantiated narrative of *Ae. albopictus* as an anthropophilic mosquito. This may be the case, but the burden of proof has not yet been reached.

Only three studies have investigated *Ae. albopictus* host preference to date. They suggest a preference for humans over other animals; however, they do not provide convincing arguments for anthropophily due to methodological and geographic restrictions. In Thailand, *Ae. albopictus*

biting collections comparing attraction to human, pig, dog, buffalo, and chicken found that human was most attractive and chicken least attractive [90]. However, in that study the experimental design may have impacted results – humans collected mosquitoes from themselves while mosquitoes were collected from other animals by another human. Additional experiments were conducted using traps, demonstrating attraction to a wide range of non-human animals. In La Réunion, no-choice (human, dog, cow, chicken, duck, shrew, rat, pig, mouse, goat, gecko, and chameleon) and choice (human, chicken, goat, and dog) experiments were conducted [27]. Without a choice, chicken was fed upon most often, followed by human and dog. When provided paired choices, human was always preferred. However, host defenses were not controlled for and likely influenced results. A third study assessed preference in Brazil, finding that humans were most often approached by *Ae. albopictus* compared with bird, cow, and dog [91] – however, lack of methodological details and absence of replication and statistical analysis prevent further interpretation of results.

These preference studies do not reveal whether variation in *Ae. albopictus* preference is a driver of variability in feeding patterns – only anthropophagic populations have been assessed thus far. Thai *Ae. albopictus* from these studies demonstrated highly anthropophagic behavior [4] and no blood meal analysis has been conducted in La Réunion, although high levels of chikungunya transmission by *Ae. albopictus* there suggest anthropophagic behavior [92]. No preference assessment has been conducted on populations with lower levels of anthropophagy. Additional investigation of *Ae. albopictus* feeding preferences should be conducted with geographically diverse populations to determine whether genetic variability of preference exists within the species. Considering high levels of genetic diversity among *Ae. albopictus* generally [93], host preference may be subject to similar levels of diversity.

One pathway by which mosquito feeding preference can evolve is through selection resulting from fitness benefits after feeding on a particular host's blood [94]. There is no evidence that *Ae. albopictus* feeding patterns are influenced by fitness amongst mammals [87, 95]; however, it may achieve superior fitness after feeding on mammals compared with birds [95, 96]. Additional studies are needed to confirm these observations. However, absence of fitness benefits between mammal hosts does not mean that host preference amongst mammals does not exist. For example, *An. gambiae* does not experience fitness benefits from feeding solely on human blood compared with a generalist diet, but has a clear preference for humans [97].

Together, these assessments of *Ae. albopictus* feeding patterns and preference suggest anthropophagy (due to high human feeding levels across many studies) and opportunism (due to high host diversity levels across many studies), but not necessarily anthropophily. Future work is necessary to understand the degree to which *Ae. albopictus* prefers humans over other mammals or animal classes. This work will be essential to clarify whether *Ae. albopictus* is anthropophilic, mammalophilic, or generalist.

Concluding Remarks

Mosquito blood feeding is a critical behavior with major implications for pathogen transmission and vector control. This behavior is the sum of innate feeding preference and external conditions that influence feeding patterns, such as host availability. Preference can be assessed through numerous experimental designs for choice and no choice assays, whereas patterns in nature are revealed through blood meal analyses. In both cases, care must be taken to avoid bias and accurately interpret results, with attention to the possibility that variability may exist between

and among populations. Use of correct terminology to describe patterns and preference is important to understand transmission risk. More research is necessary to inform optimal frameworks for determining mosquito blood feeding patterns and preferences (see **Outstanding Questions**). Here, we presented *Ae. albopictus* as a case study, illustrating how certain assumptions and incomplete evidence can lead to inconsistent interpretations about a mosquito's role as a vector. Recognizing the challenges of conducting field work on mosquito feeding behavior, we have provided suggestions for how investigators may approach patterns and preferences studies.

Outstanding Questions

- To what extent do biases influence feeding pattern results? How do under-studied biases, such as the impact of collection method impact results?
- What role does host defensive behavior play in modulating vector-borne pathogen transmission dynamics?
- At what point can a mosquito be considered anthropophagic or anthropophilic? We have provided a suggested framework; however, modeling should be employed to refine these guidelines.
- Is *Ae. albopictus* anthropophilic, mammalophilic, or generalist?
- What forces drive the evolution of feeding preferences?
- What level of heterogeneity in host preferences exist within a mosquito species?
- Which combination of preference assay characteristics provide the best reflection of true preference?
- Which host odors can enhance trapping for under-studied mosquito vectors?

- Do laboratory colonized mosquitoes evolve different host preferences and feeding behaviors?
- To what extent do mosquitoes forego feeding on a non-preferred host while waiting to feed on a preferred host?
- Is there a time point/ body condition at which a mosquito will attempt to feed on a non-preferred host in lieu of continued waiting for a preferred host (if at all)?
- How do spatial distributions of mosquito resting sites, host locations, and flight paths influence host contact and feeding patterns?

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Glossary

Anthropophilic: Prefers to feed on humans compared to other species of animal.

Anthropophagic: Feeds on humans.

Choice Experiment: Requires a choice between two hosts, allowing direct assessment of relative attraction. Individual choices are measured – when responses are aggregated, host preference can be concluded.

Definitive Host: Organism that supports the adult stage and sexual reproduction of a parasite.

Endophagic: Feeds indoors.

Exophagic: Feeds outdoors.

Feeding patterns: The actual host blood feeding that occurs in the field at a given time and place. Influenced by both extrinsic (e.g. host availability) and intrinsic (e.g. host preference) factors.

Forage Ratio: Feeding metric that compares the relative propensity for successful blood feeding on all hosts in the population by dividing the proportion of all blood meals that were taken from an animal by the proportion that that animal comprises of the full host population.

Generalist: Does not have a host preference.

Host choice: Selection of a host by an individual mosquito.

Host Feeding Index: Feeding metric that compares the relative propensity of a species to successfully feed on two animals by dividing the proportion of blood meals taken from each animal by the proportion of individuals of each species per geographic unit.

Host Preference: The tendency of mosquito species to select certain hosts over others. Measured by frequency at which individual mosquitoes make a certain choice. A solely intrinsic trait.

Mammalophilic: Prefers to feed on mammals compared to other classes of animal.

No Choice Experiment: Measures attraction of individual mosquitoes to a host in the absence of other available animals. When individual responses to a host are aggregated and compared with responses to other hosts, host preference can be concluded.

Non-peridomestic: Not in or around a human residence (e.g. farms, public land).

Opportunistic: Feeds readily on a diverse array of host animals based on availability.

Ornithophilic: Prefers to feed on birds compared to other classes of animal.

Peridomestic: In or around a human residence.

Zoophagic: Feeds on non-human animals.

Zoophilic: Preferentially feeds on non-human animals compared to humans.

Zooprophylaxis: Control strategy in which non-reservoir animals are utilized to draw host seeking vectors away from humans, reducing opportunities for transmission.

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Supplementary file:

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Box S1. Search strategy to identify* Aedes albopictus *blood meal studies

Goal: Find all published papers that report identification of field-collected *Aedes albopictus* blood meals with a reasonably representative sample size.

Search Strategy: The following two search strategies were used in the *Web of Science* (Clarivate Analytics) database:

1) TI = (("blood feed*" OR "blood-feed*" OR "bloodmeal*" OR "host feed*" OR "host-feed*" OR "blood meal*" OR "bloodfeed*" OR "host preference*" OR "feeding preference*" OR "host blood" OR "blood host*") AND ("Aedes albopictus" OR "Ae. albopictus" OR "Stegomyia albopicta" OR "Asian Tiger Mosquito*" OR "mosquito*"))

2) TS = (("blood feed*" OR "blood-feed*" OR "bloodmeal*" OR "host feed*" OR "host-feed*" OR "blood meal*" OR "bloodfeed*" OR "host preference*" OR "feeding preference*" OR "host blood" OR "blood host*") AND ("Aedes albopictus" OR "Ae. albopictus" OR "Stegomyia albopicta" OR "Asian Tiger Mosquito*"))

The search was initially conducted on 3/4/2018, updated on 12/9/2018 for papers published in 2018, and on 3/9/2021 for 2019-2021. A total of 897 citations were transferred to EndNote.

Duplicates were removed and the following inclusion/exclusion criteria were applied to the remaining papers:

Inclusion	**Exclusion**
Identified host(s) of field-caught blood fed mosquitoes	Did not identify host(s) of field-caught blood fed mosquitoes
Included at least one blood fed *Aedes albopictus*	Did not include at least one blood fed *Aedes albopictus*
Did not capture blood fed mosquitoes in live host-baited traps	Did capture blood fed mosquitoes in live host-baited traps
Sample size ≥ 60	Sample size < 60

Results: After screening titles, abstracts, and full papers according to the first three criteria, 36 papers reporting *Aedes albopictus* blood meal(s) were found. Of these, only 17 had a sample size over 60. An additional two recently published papers were included that were not yet available on Web of Science, for a total of 19 studies reviewed here.

Box S2. Working towards a Terminology Threshold

Creating threshold values for consistent application to mosquito blood feeding terminology is difficult due to the unique nature of each species and disease system. We began our thought process for creating meaningful thresholds by considering the blood feeding term in the context of pathogen transmission. The exercise is complicated because pathogen transmission is reliant on several factors in addition to daily blood feeding rate. Consequently, there is no general feeding rate threshold that will result in significant pathogen transmission across all

transmission contexts. Furthermore, "significant transmission level" itself is subjective. For example, is one human case per year significant? Is 100? The answer may depend on infection severity.

We acknowledge the complexity of creating thresholds with epidemiological significance across all systems. However, we believe it is important to create a standard threshold so that terms are used consistently in the literature with clearer meaning.

As a thought exercise, we first considered zoonotic diseases. We considered what level of human feeding (anthropophagy) would result in the potential for an individual mosquito to serve as a human bridge vector. Using the vectorial capacity framework, we assume that, on average, a mosquito would need to feed two times in its life to transmit a pathogen: it must become infected with the first meal and transmit the pathogen in a subsequent meal after the extrinsic incubation period (EIP). Considering the duration of EIP for many important arboviruses, the mosquito is likely to take a blood meal in the middle of the EIP. This is a major assumption due to the variability in biting frequency and the likelihood that many mosquitoes die before reaching the end of the EIP – these estimates warrant more field-based research. Given three relevant meals in a lifetime (one infectious meal, one intermediate, and one meal following the EIP), zoonotic pathogen transmission to a human host would require the first infectious meal to be from a reservoir animal and the post-EIP meal to be from a human. Therefore, a gonotrophically concordant mosquito must take at least 33% of its meals from a human to serve as a bridge vector. Thus, we suggest that 33% of blood meals from a given host species or class as a threshold estimate. Any feeding above this level would make a mosquito species -phagic.

We then considered a framework that would make -philic (preference) terms maximally useful. We concluded that establishing preference would be most helpful in the context of

identifying potential amplifying vectors. We can thereby use -phily as a framework to consider when a vector would choose to feed often enough on the reservoir host to contribute to on-going transmission, given equal availability of hosts. We first considered an anthroponosis and again assumed three blood meals. The first infectious blood meal must be from a human followed by at least one human meal post-EIP. Therefore, an individual mosquito must choose a human 2/3 times to potentially transmit an anthroponotic pathogen (given equal availability of hosts). Therefore, if an individual chooses a human 66.6% of the time and a non-human animal 33.3% of the time, it has the potential to vector an anthroponotic pathogen. Thus, we suggest that -philic terms be reserved for species that are at least two times more likely to feed on a given host species/class compared with an alternative host.

The suggested thresholds are based on a number of assumptions and make large generalizations. We acknowledge these limitations but provide some guidance towards a common framework with which to use these terms. Even an imperfect threshold will provide clarity and a common language with which to discuss vector feeding behavior. Measuring relevant field parameters and their variation (i.e., survival rate, longevity and biting rate) for certain important species where this data is lacking, combined with robust quantitative modeling will be important future contributions to the application of mosquito blood feeding terminology and our understanding of pathogen transmission dynamics.

TABLE 1.3: FIELD-COLLECTED *AEDES ALBOPICTUS* BLOOD MEAL IDENTIFICATION STUDIES

First Author	Year	Title	Location	Sample Size
Tempelis [S1]	1970	Blood feeding habits of four species of mosquito found in Hawaii	Hawaii, USA	538
Sullivan [S2]	1971	Observations of the host range and feeding preferences of *Aedes albopictus* (Skuse)	Thailand	3
Cully [S3]	1991	Antibodies to La Crosse virus in eastern chipmunks in Indiana near an *Aedes albopictus* population	Indiana, USA	6
Savage [S4]	1993	Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) at a Temperate North American site	Missouri, USA	139
Niebylski [S5]	1994	Blood hosts of *Aedes albopictus* in the United States	Missouri, Florida, Indiana, Illinois, and Louisiana, USA	245
Gomes [S6]	2003	Host feeding patterns of potential human disease vectors in the Paraíba Valley Region, State of Sao Paulo, Brazil	State of Sao Paulo, Brazil	177
Gingrich [S7]	2005	Host-feeding patterns of suspected West Nile Virus mosquito vectors in Delaware, 2001-2002	Delaware, USA	22
Ponlawat [S8]	2005	Blood Feeding Patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand	Thailand	105
Richards [S9]	2006	Host-Feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of Central North Carolina	North Carolina, USA	1094
Dennett [S10]	2007	Associations between two mosquito populations and West Nile Virus in Harris County, Texas, 2003-06	Texas, USA	26
Kim [S11]	2009	Bloodmeal identification and detection of avian malaria parasite from mosquitoes (Diptera: Culicidae) inhabiting coastal areas of Tokyo Bay, Japan	Tokyo Bay, Japan	13
Sawabe [S12]	2010	Host-feeding habits of *Culex pipiens* and *Aedes albopictus* (Diptera: Culicidae) collected at the urban and suburban residential areas of Japan	Japan	114
Valerio [S13]	2010	Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in Urban and Rural contexts within Rome Province, Italy	Rome Province, Italy	303
Munoz [S14]	2011	Host-Feeding Patterns of Native *Culex pipiens* and Invasive *Aedes albopictus* Mosquitoes (Diptera: Culicidae) in Urban Zones from Barcelona, Spain	Barcelona, Spain	30
Haddad [S15]	2012	*Aedes albopictus* in Lebanon, a potential risk of arboviruses outbreak	Lebanon	23
Kamgang [S16]	2012	Notes on the blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae)	Cameroon	162
Tuten [S17]	2012	Blood-feeding ecology of mosquitoes in zoos	South Carolina, USA	5
Egizi [S18]	2013	Rapid blood meal scoring in anthropophilic A*edes albopictus* and application of PCR blocking to avoid pseudogenes	New Jersey, USA	79
Tanigawa [S19]	2013	Molecular identification of avian *Haemosporidia* in Wild Birds and Mosquitoes on Tsushima Island, Japan	Tsushima Island, Japan	6
de Carvalho [S20]	2014	Blood meal sources of mosquitoes captured in municipal parks in Sao Paulo, Brazil	Sao Paulo, Brazil	4
Faraji [S21]	2014	Comparative host feeding patterns of the Asian Tiger Mosquito, *Aedes albopictus,* in urban and suburban Northeastern USA and implications for disease transmission	New Jersey, USA	165

Guo [S22]	2014	Host-feeding patterns of mosquitoes in a rural malaria-endemic region in Hainan Island, China	Hainan Island, China	138
Kek [S23]	2014	Feeding Host Range of *Aedes albopictus* (Diptera: Culicidae) demonstrates its opportunistic host-seeking behavior in rural Singapore	Singapore	~107 (grouped)
Khaklang [S24]	2014	Species composition and blood meal analysis of mosquitoes collected from a tourist island, Koh Chang, Thailand	Koh Chang, Thailand	8
Samuel [S25]	2014	Dengue vectors prevalence and the related risk factors involved with dengue in Thiruvananthapuram district, Kerala, south India	Kerala, India	Not reported
Martinez-de la Puente [S26]	2015	Avian malaria parasites in the last supper: identifying encounters between parasites and the invasive Asian mosquito tiger and native mosquito species in Italy	Italy	34
Sivan [S27]	2015	Host-feeding patterns of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in heterogeneous landscapes of South Andaman and Nicobar islands, India	India	91
Westby [S28]	2015	La Crosse Encephalitis Virus infection in field-collected Aedes albopictus, Aedes japonicus, and Aedes triseriatus in Tennessee	Tennessee, USA	11
Borstler [S29]	2016	Host-feeding patterns of mosquito species in Germany	Germany	1
Kim [S30]	2017	Host-feeding pattern and dengue virus detection in Aedes albopictus (Diptera: Culicidae) captured in an urban park in Korea	Korea	90
Goodman [S31]	2018	Primary blood-hosts of mosquitoes are influenced by social and ecological conditions in a complex urban landscape	Maryland, USA	177
Stenn [S32]	2019	Vertebrate Hosts of Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus (Diptera: Culicidae) as Potential Vectors of Zika Virus in Florida	Florida, USA	68
Martínez-de la Puente [S33]	2020	Mosquitoes in an Urban Zoo: Identification of Blood Meals, Flight Distances of Engorged Females, and Avian Malaria Infections	Spain	3
Mann [S34]	2020	Feeding habits of vector mosquitoes in Harris County, TX, 2018	Texas, USA	5
Martínez-de la Puente [S35]	2020	Do Invasive Mosquito and Bird Species Alter Avian Malaria Parasite Transmission?	Spain	20
Young [S36]	2020	Identification of Mosquito Bloodmeals Collected in Diverse Habitats in Malaysian Borneo Using COI Barcoding	Malaysian Borneo	16
Little [S37]	2021	Host interactions of *Aedes albopictus,* an invasive vector of arboviruses, in Virginia, USA	Virginia, USA	961
Fikrig [S38]	2021	The effects of host availability and fitness on *Aedes albopictus* blood feeding patterns in New York	New York, USA	90

TABLE 1.4: METHODOLOGY AND RESULTS OF AEDES ALBOPICTUS BLOOD MEAL IDENTIFICATION STUDIES WITH SUFFICIENT SAMPLE SIZE

Author	Year	Location	Site Type	Blood Analysis Method	Collection Method	Sample Size	Human	Dog	Cat	Deer	Horse	Bovine	Pig	Rabbit	Squirrel/Chipmunk	Raccoon	Rodent	Unknown mammal	other mammal	Opossum	Mammal Total %	Bird	Reptile	Amphibian	Fish	Number species	Notes
Tempelis	1970	Hawaii, USA	Both	Precipitin	Resting	538	18.1	19.8	10.2	*	6.6	17.9	8.1	0.2	*	*	*	5	8.3	*	94.2	5.8	*	0	*	11+	Mongoose, chicken, passeriformes, booby
Savage	1993	Missouri, USA	Non-peridomestic	Precipitin and ELISA	Resting and Host-seeking	139	6.5	10.8	0	11.5	0	0.7	0	19.4	5.8	0.7	2.9	17.3	*	3.6	79.2	20.9	0	*	*	13+	Passeriformes, columbiformes, ciconiiformes, quail, unidentified bird (4+ bird species)
Niebylski	1994	MO, FL, IN, IL, and LA, USA	Non-peridomestic	Precipitin and ELISA	Resting	245	4.1	5.7	0.8	4.1	0	5.3	0	37.1	2.9	1.2	30.4	5.7	*	0	97.3	1.2	2	*	*	11+	
Gomes	2003	Brazil	Peridomestic	Precipitin	Resting	177	50.2	2.5	0.4	*	1.7	6.4	1.7	*	*	*	10.6	*	*	*	73.5	1.7	*	*	*	8+	
Ponlawat	2005	Thailand	Peridomestic	ELISA	Resting	105	100	0.95	0.95	*	*	0	3.8	*	*	*	*	*	*	*	100	0	*	*	*	4+	
Richards	2006	North Carolina, USA	Peridomestic	ELISA and PCR on subset	Resting	1094	20	11.3	17.6	2.3	3.7	*	*	9.5	8.6	4.8	*	*	*	5.8	83.6	7.5	1.6	1.8	*	14+	0.09% fed on cardinal and 0.2% on white pelican-like bird species; 1.45% on chicken
Sawabe	2010	Japan	Peridomestic	PCR	Resting and Host-seeking	114	64.9	0	7.9	0	0	7.9	0	0	0	0	14	0	*	0	94.7	6.1	0.5?	0.5?	0	7+	at least 2 species of bird (1 was unidentified bird); 0.5% was for combined amphibian and reptile, but it's unclear how many of each
Valerio	2010	Italy	Both	ELISA	Gravid	303	80.9	6.6	8.3	*	5.9	6.3	*	0.7	*	*	0.3	*	*	*	109	3	*	*	*	8+	
Kamgang	2012	Cameroon	Non-peridomestic	ELISA	Resting	162	99.4	0	*	*	0		1.9	*	*	*	*	*	*	*	101.3	0	1.9	*	*	3	
Egizi	2013	New Jersey, USA	Peridomestic	PCR	Host-seeking	79	69.6	10.1	15.2	1.3	0	0	0	2.5	3.8	0	1.3	*	0	3.8	107.6	0	*	*	*	8	Only a subset was tested with bird primers; did not test with primers that would detect reptiles
Faraji	2014	New Jersey, USA	Peridomestic	PCR	Host-seeking	165	58.2	14.5	23	0.6	0	0	0	1.2	3.6	0	0.6	*	0	4.2	105.9	0	0	0	0	8	
Guo	2014	China	Both	PCR	Resting and Host-seeking	138	66.7	12.3	0	0	0	3.6	9.4	0	0	0	0	*	0	0	92	8	0	0	0	6	
Kek	2014	Singapore	Both	PCR	Resting and Host-seeking	~107	85	1.9	0.9	0	0	0	1.9	0	0	0	0	*	3.7	0	93.4	0	0.9	0	0	7	Sample size is not precise because they did pools of 10 for mosquitoes that did not have visible blood meal, so this could be an under-estimate; 3.7% shrew; 0.9% turtle
Sivan	2015	India	Both	Precipitin	Host-seeking	91	56	*	*	*	*	20.9	4.4	*	*	*	2.2	*	11	*	94.5	5.5	*	*	*	6	goat
Kim	2017	Korea	Non-peridomestic	PCR	Host-seeking	90	61.1	0	5.6	0	0	0	0	0	0	0	3.3	*	2.2	0	72.2	25.6	0	2.2	1.1	13	bat
Goodman	2018	Maryland, USA	Peridomestic	PCR	Host-seeking	177	13.6	1	12.4	<1	0	0	0	0	0	0	72.3	*	0	0	100	0	0	0	0	5	
Stenn	2019	Florida, USA	Both	PCR	Host-seeking	67	91	0	0	0	0	0	0	3	0	1.5	1.5	*	1.5	0	98.5	0	1.5	0	0	6	Authors report a sample size of 68 but only provide 67 identifications; armadillo
Little	2021	Virginia, USA	Both	PCR	Host-seeking	961	7.3	2.3	50.5	12.3	0	0	0	1.3	2	0.2	2.9	*	0.2	17.2	96.2	0.3	3.9	0	0	10	fox, 2 species of birds: American robin (0.2) and common grackle (0.1); 3 species of reptile: Common box turtle (3.5), Eastern box turtle (0.2), common musk turtle (0.1)
Fikrig	2021	New York, USA	Peridomestic	PCR	Resting	90	32.2	5.6	24.4	0	17.8	0	0	1.1	1.1	1.1	1.1	*	2.2	13.3	100	0	0	0	0	15	goat

Please note that percentages may not add to 100 within a study because some studies include mosquitoes with multiple meals. E.g. if a mosquito fed on a human and a dog, that sample is included in both the dog and human percentages to reflect the proportion of mosquitoes that fed on a given animal.

Notes provide additional information, such as abnormalities in reporting, species identifications of less common animals included in "other mammal", "Bird", "Reptile", or "Amphibian"

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Chapter 2 : The effects of host availability and fitness on *Aedes albopictus* blood feeding patterns in New York[*]

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Key Words: blood meal, mosquito, survival, fecundity, aspirator collections

Abstract

Aedes albopictus is a competent vector of numerous pathogens, representing a range of transmission cycles involving unique hosts. Despite the important status of this vector, variation in its feeding patterns is poorly understood. We examined the feeding patterns of *Ae. albopictus* utilizing resting collections in Long Island, New York, and contextualized blood meal sources with host availability measured by household interviews and camera traps. We identified 90 blood meals, including 29 human, 22 cat, 16 horse, 12 opossum, 5 dog, 2 goat, and 1 each of rabbit, rat, squirrel and raccoon. This is only the third study of *Ae. albopictus* blood feeding biology that quantitatively assessed domestic host availability and is the first to do so with wild animals. Host feeding indices showed that cats and dogs were fed upon disproportionately often compared with humans. Forage ratios suggested a tendency to feed on cats and opossums and to

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avoid raccoons, squirrels, and birds. This feeding pattern was different from another published study from Baltimore, where *Ae. albopictus* fed more often on rats than humans. To understand if these differences were due to host availability or mosquito population variation, we compared the fitness of New York and Baltimore *Ae. albopictus* after feeding on rat and human blood. In addition, we examined fitness within the NY population after feeding on human, rat, cat, horse, and opossum blood. Together, our results do not indicate major mosquito fitness differences by blood hosts, suggesting that fitness benefits do not drive Northeastern *Ae. albopictus* feeding patterns.

Introduction

Aedes albopictus is a widely invasive mosquito of human and veterinary health importance. This species is capable of transmitting over 20 pathogens in laboratory assays[1], and is a confirmed natural vector of dengue, Zika, and chikungunya viruses, and dog heartworm[1, 2]. Additionally, virus detection in field-collected mosquitoes has led *Aedes albopictus* to be a suspected vector of numerous additional pathogens, including eastern equine encephalitis and West Nile due to virus detection in field-collected mosquitoes, although to date, there is no direct evidence of transmission to humans[1]. These pathogens encompass vastly different transmission cycles. Some are anthroponoses, transmitted from human to mosquito (e.g. Zika virus), while others are zoonoses, transmitted from non-human animals to mosquitoes (e.g. dog heartworm). Transmission of these zoonoses may occasionally result in human infection (e.g. West Nile virus). In light of the broad vector potential of *Ae. albopictus* and variation in feeding patterns in nature, it is critical to perform host feeding studies in locations relevant to human and animal health risk.

Variation in mosquito host feeding patterns can be influenced by a number of factors including innate host preference, environmental conditions, host availability, and the design of the studies themselves[3, 4]. These factors may explain the variability in host feeding patterns reported for *Ae. albopictus* in the literature, which range from generalist or mammalophagic to highly anthropophagic (=human feeding). For example, a high percentage of mosquitoes with human-derived blood meals were identified in tropical countries such as Thailand (100%) and Cameroon (99.4%)[5, 6]. In Thailand, aspirator collections were conducted around human dwellings, however, in Cameroon, mosquitoes were collected at a leisure and equestrian center, both of which were surrounded by human dwellings. In some parts of the USA, human feeding frequency was much lower, such as at a tire dump in Missouri (6.5%), urban Baltimore, Maryland (13.6%), urban and rural sites in Hawaii (18.1%) and Virginia (7.3%), and suburban North Carolina (20%)[7-11]. Additional studies have reported moderate human feeding rates such as in urban and peripheral sites in Brazil, urban and suburban Japan, and suburban New Jersey, USA[12-14]. Of those populations that did not feed predominantly on humans, most fed on a diverse array of animals, with the exception of Baltimore, where a striking number of *Ae. albopictus* fed on rats (72.3%)[7].

One notable consistency amongst all published studies (with a sample size over 60) is a tendency for *Ae. albopictus* to feed primarily on mammals compared with birds and reptiles[5-21]. About half of studies report feeding on birds at low rates (1.7% to 25.6% of all blood meals)[8, 10-13, 15, 17, 20, 22]. A tendency to feed even sporadically on birds is particularly important because of their role as amplifying hosts of arboviruses such as West Nile and Eastern equine encephalitis.

Host availability is rarely considered in the design of mosquito blood meal collection studies conducted in the field despite its importance in driving mosquito blood feeding patterns

and thus interpreting study results. In Italy, *Ae. albopictus* from urban and rural sites had replicable differences in feeding patterns, mirroring differences in host availability at these sites[15]. Similar observations were made in Singapore and India[22, 23]. However, the authors only described the site qualitatively (e.g. rural vs. urban) and did not quantify host availability. We are aware of only two published studies (in North Carolina and Brazil) that have quantitatively assessed the link between host availability and blood feeding for *Ae. albopictus*[11, 13]. Results from these two studies do not provide a clear picture of whether *Ae. albopictus* feeds disproportionately often on humans compared with other mammals, with results varying depending on measurement type (abundance vs. time-weighted), stratification level (household vs. hectare), and which non-human animals were included in the paired comparison.

In addition to host availability, host attraction may also influence blood feeding patterns[24]. Only two published studies have explored host attraction in *Ae. albopictus*[25, 26]. The authors of both studies reported higher attraction to humans compared with numerous other hosts including dogs and chickens. Preferential attraction to hosts is determined genetically, and may evolve as a result of elevated mosquito fitness after ingesting a given species' blood[24, 27]. This has been demonstrated for *Ae. aegypti*, which maximizes reproductive fitness on human blood, its preferred host[28]. Only two studies have addressed the impact of blood from different host species on *Ae. albopictus* egg production[29, 30], but none have compared both survival and fecundity following blood meals from the most ecologically relevant hosts.

We sought to determine *Ae. albopictus* feeding patterns in select suburban and farm landscapes along its front of active northward expansion in New York (NY) State[31]. Our aim was to investigate feeding patterns in the context of host availability and consequences for mosquito fitness. Ultimately, we wanted to fill a gap in our understanding of *Ae. albopictus* feeding

ecology along its Northeast USA range limit and how it might relate to public health risk. To meet our objectives, we performed host censuses for use in calculating host feeding indices and forage ratios. We then assessed whether fitness of NY *Ae. albopictus* varied by host blood source ingested under laboratory conditions through a series of life table studies. To explore potential population-level differences, we compared fitness of *Ae. albopictus* individuals from NY and Baltimore after ingesting human and rat blood meals.

Methods

Field Sites

Eight sites were selected in Suffolk County on Long Island, NY (Figure 2.1): four farms and four residential areas, each containing between nine and seventeen collection properties. *Ae. albopictus* has been present in Suffolk County since 2004, although its distribution is not uniform or complete across the county (Moses Cucura, pers comm). Residential sites were selected based on *Ae. albopictus* presence reported by the Suffolk County Vector Control and Arthropod-Borne Disease Laboratory and larval distribution data[32]. All residential sites were suburban, with variable human population density: Central Islip (1,853 people/sq km), Bay Shore (1,853 people/sq km), Babylon (1,660 people/sq km), and Hauppauge (734 people/sq km). All four farms were partially bordered by suburban residential and forested natural landscapes. One was a petting zoo and three were riding stables.

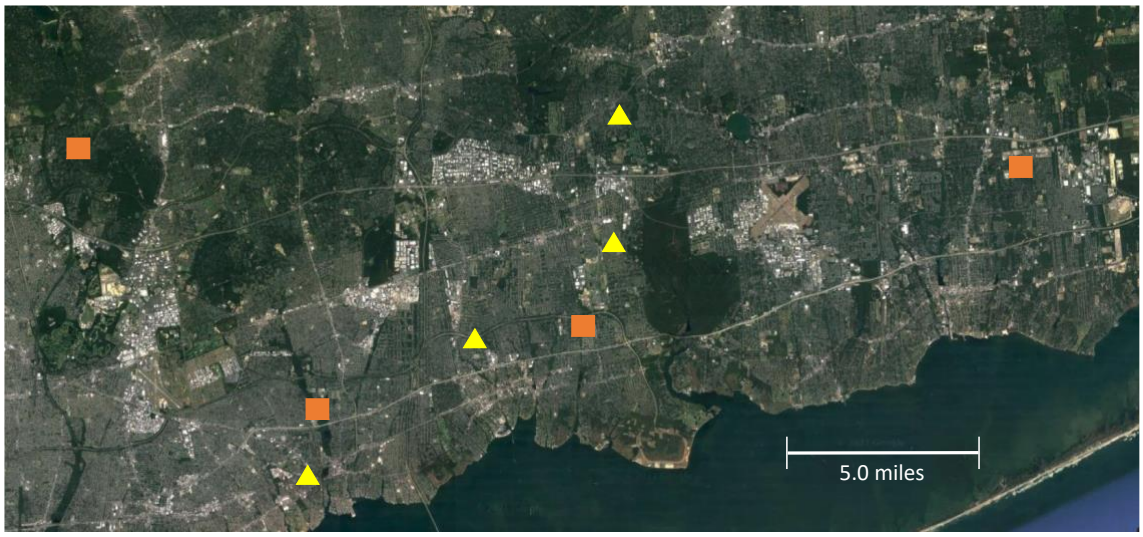

FIGURE 2.1: SITE MAP

Google Earth satellite image of Long Island (as displayed in June 2021). Yellow triangles represent residential sites and orange squares represent farm sites.

Mosquito Collection

Weekly collections were conducted at each site between 20 June and 15 August, 2018 with large custom-designed aspirators (30.5 cm diameter, 114 cm height, 12 V PM DC 2350 RPM, 1/35 horsepower, 3.7 amp motor)[5]. Collections were conducted once per week at each site between 08:00 and 19:00 hrs, with two sites visited per day (one in the morning, one in the afternoon). Morning and afternoon visitation was typically rotated from week to week. Two teams of three researchers worked simultaneously at separate properties at residential sites and together at farm sites. Two aspirators were operated per team for the length of time necessary to sample the full property (most collection times were between 7-12min; range from 2.5-17min). Mosquitoes were immobilized in acetone-treated jars (3 min) and sorted in the field to remove non-mosquito by-catch. The samples were transported on ice to the laboratory for identification according to a taxonomic key[33]. *Aedes albopictus* were considered engorged if blood was visible in the

abdomen upon examination. Mosquitoes were stored at -20°C and transported to Cornell University on dry ice for blood meal identification.

Blood Meal Identification:

Abdomens were removed from mosquitoes using forceps and transferred to sterile microcentrifuge tubes. To avoid cross-contamination, forceps were dipped in ethanol[34] and flame-sterilized [35] between each sample. DNA was extracted from abdomens using Qiagen Puregene Cell kit (Qiagen Sciences, Germantown, MD, USA). To identify blood meals, we amplified templates from the vertebrate-specific cytochrome c oxidase subunit I (*COI)* "barcoding" gene. Primers designed by Reeves et al. (2018) were used to amplify a 395 base pair amplicon[36] (Table 2.1).

TABLE 2.1: PRIMER SEQUENCES DESIGNED BY REEVES ET AL. (2018)

Primer Name	Sequence
VertCOI_7194_F	5'- CGM ATR AAY AAY ATR AGC TTC TGA Y -3'
Mod_RepCOI_R	5'- TTC DGG RTG NCC RAA RAA TCA -3'

Other Reeves *COI* primers were not used due to co-amplification of *Ae. albopictus* DNA. Co-amplification is a recurrent issue with identifying *Ae. albopictus* blood meals due to matching sequences between its own genome and primers designed for use in blood meal studies of other mosquito species[16]. Notably, cytochrome b primers designed by Egizi et al. (2013) were used initially, but due to a low success rate in our hands, we switched to the Reeves primers[36]. Three blood meals identified with the Egizi primers were not successfully amplified by the Reeves primers; results with both primer sets were combined for our data analysis.

PCR conditions were slightly modified from Reeves et al. (2018) in order to minimize co-amplification of *Ae. albopictus* DNA and maximize amplification of the desired amplicon[36]. Reactions were performed with total volume of 20 μL, consisting of 10 μL of 2.0X Apex Taq RED Master Mix (Genesee Scientific Corp., San Diego, CA), 0.75 μL of VertCOI_7194_F forward primer (10 μM), 0.75 μL of Mod_RepCOI_R reverse primer (10 μM), 6.5 μL sterile nuclease-free H_2O, and 2 μL of extracted DNA. Most reactions were conducted with the following thermocycling conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 40 s, 53.5°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 7 min. The annealing temperature was modified from Reeves et al. (2018) in order to minimize amplification of *Ae. albopictus* DNA according to a temperature gradient test conducted on positive (human-fed) and negative (non-fed) mosquito controls. Conditions were further modified for a subset of reactions to optimize amplification: 94°C for 3 min, followed by 5 cycles of 94°C for 40 s, 45°C for 30 s, and 72°C for 60 s, and then 35 cycles of 94°C for 40 s, 48.5°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 7 min. All reactions were conducted alongside a positive (human-fed mosquito) and negative (sterile nuclease-free water) control. PCR products (5 μL) were loaded onto a 1% agarose gel stained with gelRED, electrophoresed, and visualized with UV light (Mighty Bright, Hoefer Scientific Instruments, San Francisco, CA, USA).

Samples with positive bands after gel electrophoresis were purified with FastAP and Exonuclease (ThermoFisher Scientific, Waltham, MA, USA) and submitted for Sanger sequencing at the Cornell University Biotechnology Resources Center. Sequences were compared with the available database in NCBI Basic Local Alignment Search Tool (BLASTn) and were identified to a source if matches were ≥98% with a sequence of known origin (with the exception of an eastern gray squirrel (*Sciurus carolinensis*) sequence, which had a 95.5% match).

Host Availability

Household Interviews:

To estimate domestic host availability, household interviews were conducted weekly at time of collection. Interviews were conducted by trained field collection staff with a set of uniform questions (see Supplemental Materials S1). Interviewers were typically rotated between houses to further reduce interviewer bias. Residents were asked about the number of people and pets living in their house and the amount of time each host type spent outside that day and the two days prior. This time frame was investigated because digestion may prevent blood meal identification at approximately 48 hours after feeding under field relevant temperatures[37]. Interviews were conducted in English or Spanish depending on homeowner preference.

Camera Traps:

To estimate wild host availability, two motion-triggered camera traps (Moultrie M-880, #MCG-12691, Calera, AL, USA) were set at each site as soon as they were available, from 16 July to 13 August 2018, on selected properties in residential sites and different locations within farm sites. Cameras were operated according to the specifications described by Linske et al.[38], with the exclusion of scent lures: 30-s detection delay between images, high passive infrared sensitivity, single still-image photo, 1.0 m above ground, and slight downward angle to capture both small and large hosts. Camera data were used to estimate host abundance by host type by determining the number of animal encounters with the camera per trap day. If a given host type was photographed within 30 min of the last image of that animal, it was considered the same individual and was not counted separately. If multiple individuals were captured in one image within 30 min of last sighting, the count was equal to the maximum number captured together in an image.

Fitness by Host Blood Source

Mosquito Rearing:

Aedes albopictus eggs were collected from five towns in NY (3 on Long Island and 2 in the Hudson Valley region) for a previous study[39], including two of the residential sites studied here (see Supplemental Materials S2 for colony information). Each location was reared separately in colony for a few generations and then combined into one large NY colony, totaling six to ten generations of laboratory rearing prior to use. Eggs from Baltimore, MD (between F_3 and F_6 depending on replicate) were reared synchronously with the NY colony in order to assess between population differences. For each replicate, eggs were vacuum hatched, provided with a pinch of pulverized fish food (crushed Cichlid Gold™ fish food pellets; Hikari, Himeji, Japan), and one day later, separated into trays of 200 larvae, with 1L of distilled water, and 4 Cichlid Gold™ fish food pellets. Adult mosquitoes were maintained in an environmental chamber (28°C, 71.9% ± 9.5% relative humidity, 10 hr light, 10 hr dark, 2 hr dusk/dawn). Cups of 200 pupae were placed into cages inside the chamber, and upon eclosion, 10% sucrose was provided for 2-4 d. Males were removed and sucrose was replaced with distilled water for 1 d prior to blood feeding.

Blood:

Human (Lampire Biologicals; Pipersville, PA, USA), opossum (The Janet L. Swanson Wildlife Health Center; Ithaca, NY, USA), rat (The Center for Animal Resources and Education, Cornell University), cat (The Center for Animal Resources and Education at Cornell University; Ithaca, NY, USA) and horse (Lampire Biologicals; Pipersville, PA, USA) blood treated with

anticoagulant (sodium citrate) was stored at -20°C upon arrival. Blood was thawed in warm water immediately before use. Mosquito blood feeding was conducted with artificial feeders (water reservoir at 37°C and de-salted sausage casings as membrane) as described previously[40].

Within-population fitness impacts for NY Ae. albopictus

In order to determine whether fitness advantages for different host blood sources reflected the feeding pattern and level of host usage of NY *Ae. albopictus*, we assessed fecundity and survival of females after feeding on human, cat, horse, opossum, and rat blood. These blood sources were chosen based on host species identified in our blood meal analysis. For the purpose of this study, the NY colony was considered one population, although it was established with *Ae. albopictus* collected from sites across Southern NY.

Fecundity and Survival: Fully engorged mosquitoes (approximately 35 per blood source per replicate and 3-4 replicates per group) were gently transferred individually into 0.5L paper cups with a dry oviposition vessel. Mosquitoes were maintained in individual cups in the environmental chamber as described above. One day after blood feeding, strained larval rearing water was added to oviposition vessels to encourage egg laying. No additional water or sugar was provided. Each mosquito was checked daily for presence of eggs (first day of egg lay) and mortality until all females had died. Total number of eggs laid per female was recorded at the end of experiment. Dead mosquitoes were frozen at -20°C and later dissected to determine number of mature retained eggs, if any. We compared the total eggs produced (retained + laid eggs). In replicate two, mosquitoes with a large number of retained eggs were not counted and were therefore not included in the egg analyses but were included in survival analyses. For individuals where egg retention data were not available, number of eggs laid was used. The

following blood types were tested: replicate one included human, rat, cat, and horse; replicates two and three included human, rat, cat, horse, and opossum; replicate four included human, rat and opossum.

Between-population differences of NY and Baltimore Ae. albopictus

Because of the striking differences in field-collected host blood meal sources between our study and a prior Baltimore study (where *Ae. albopictus* fed more often on rats and less often on humans than in NY[7]), we assessed whether there were also differences in fitness between *Ae. albopictus* from these two locations after feeding on rat and human blood.

Fecundity and survival: NY and Baltimore *Ae. albopictus* were fed rat and human blood and observed synchronously using the methods described above. The rat and human-fed NY individual mosquitoes from replicates 1-3 of the within-population fitness assessment described above were used to compare both between-population fitness of NY and Baltimore *Ae. albopictus* and within-population fitness of NY *Ae. albopictus.* The wing lengths of a subset of NY and Baltimore individuals were measured to control for body size differences between the two population cohorts[41, 42].

Data Analysis

Host availability

Residential Host Feeding Index: Abundance and time-weighted host feeding indices (HFI) were calculated using blood meal identification data from residential areas and household interview data for humans, cats and dogs. Feeding indices were calculated according to equations described by Kay et al. (1979) and modified by Richards et al. (2006) as follows[11, 43]:

$$\mathrm{HFI} = \frac{\mathrm{B}_x/\mathrm{B}_y}{\mathrm{H}_x/\mathrm{H}_y}$$

where B_x and B_y represent the average number of blood meals from host *x* and host *y* per household and H_x and H_y represent the average number of host *x* and host *y* residing per household. Averages were calculated with data from households positive for at least one bloodmeal. Data were aggregated across all four residential sites because household and site-specific calculations frequently resulted in undefined values due to zeroes in the denominators.

A time-weighted feeding index [11] was calculated as follows:

$$\mathrm{HFI}_\mathrm{T} = \mathrm{HFI}\left(\frac{\mathrm{T}_y}{\mathrm{T}_x}\right)$$

where T_y and T_x represent the time spent outside by hosts *y* and *x*, respectively. When household interview data were missing on the date of bloodmeal collection (26 of 66 surveys), the average of all other interview responses from that household was used as an approximation.

An HFI or HFI_T greater than 1 indicated that host *x* was fed upon more often than expected compared with host *y* given their abundance or time spent outside. An HFI or HFI_T equal to 1 indicated that the hosts were fed upon in proportion to their availability and an HFI or HFI_T less than 1 indicated that host *y* was fed upon more often than expected compared with host *x*. Note that while an HFI or HFI_T greater or less than 1 may reflect *Ae. albopictus* preference, it does not conclusively demonstrate it, as we cannot rule out influences from other factors such as host defenses, timing of host availability, or host location in the yard.

Residential Forage Ratio: Forage ratios represent another method for determining host feeding frequency by host availability[5]. In our study, these were calculated using blood meal

identification data and camera trap images from residential sites. Forage ratios were calculated for each host type that was captured by camera traps as follows[44]:

$$\frac{\text{Number of blood meals from host } x \text{ / Total number of all blood meals}}{\text{Number of host } x \text{ in the population /Total number of all hosts in population}}$$

In the case of this study, the proportion of all hosts represented by host *x* was approximated by the proportion of all camera trap images that were taken of host *x*.

A forage ratio greater than one suggests that the host was fed upon more often than expected given its abundance and less than one suggests that the host was fed upon less often than expected. A forage ratio equal to one indicates that the host was fed upon in proportion to its abundance in the population. As with host feeding indices, forage ratios may reflect preference but do not prove it because the same sources of bias may impact these results.

Farm Host Availability: At the farm sites, host feeding indices and forage ratios were not calculated due to small sample sizes and technical difficulties of defining host availability, making quantification of forage ratios and feeding indices uninformative. Interviews of human and domestic animal availability were only conducted once at farms during the last week of collections. Farm owners could not accurately estimate human exposure due to unpredictable influx of people on site for riding lessons and farm work. Animal exposure could not be reliably measured because of inconsistent use of fenced paddocks and semi-enclosed barns. Camera traps were positioned in order to picture wild animals at the outskirts of the fenced paddocks and therefore did not often picture domestic farm animals. Interview and camera trap data is reported for each but are only qualitatively compared with blood meal data; no further calculations were conducted.

Life table studies- fitness by host blood source

Within-population fitness impact: The effect of host blood source on egg production (fecundity) for the NY colony was assessed with a linear model, including replicate and mosquito survival as covariates. The effect of host blood source on mosquito survival was also determined using a linear model, including replicate as a covariate. Estimated marginal means *post hoc* analyses were conducted using the emmeans package[45]. Survival curves were created with the average proportion surviving across the replicates and compared for each host blood source. The basic reproductive rate (R_0) was calculated for each blood type and replicate according to previously described equations[46]. The effect of blood type on R_0 was compared via a linear model.

Between-population differences: Egg production and survival were compared between human/rat, NY/ Baltimore groups using linear models, as described above. However, in this case, number of eggs produced by each individual was divided by average wing length of the cohort, reported as eggs per mm wing length (eggs/mm wl), in order to control for the effect of body size, which differed between Baltimore and NY colonies despite identical rearing methods.

Ethics approval:

Survey protocols were reviewed and considered exempt by Cornell University's Institutional Review Board (IRB). Blood was acquired from vendors or groups that already had appropriate permits and thus blood feeding was not regulated by the Institutional Animal Care and Use Committee (IACUC).

Results

Blood Meal Identification:

3,241 *Ae. albopictus* were collected between 20 June and 15 August (1,575 female and 1,666 male), of which 182 (14% of aspirator-collected females) were blood-fed. Of the females designated blood fed, 149 blood meals (81.9%) were between half-digested and fully engorged. An additional 6 mosquitoes were captured by hand nets with non-fresh blood while host-seeking near collectors, indicating that the bloodmeal was not taken from collectors. Host identity was successfully assigned to 90 samples (49.5%), including 29 human (*Homo sapiens;* 32.2%), 22 cat (*Felis catus;* 24.4%), 16 horse (*Equus caballus;* 17.8%), 12 opossum (*Didelphis virginiana;* 13.3%), 5 dog (*Canis lupus familiaris;* 5.6%), 2 goat (*Capra hircus;* 2.2%), and 1 each of rabbit (*Sylvilagus floridanus*; 1.1%), rat (*Rattus norvegicus*; 1.1%), squirrel (*Sciurus carolinensis*; 1.1%), and racoon (*Procyon lotor*; 1.1%). One of these was captured by hand net (with a human blood meal). When categorized by residential (n=66) or farm sites (n=24), most of the blood fed female *Ae. albopictus* from residential sampling sites indicated blood meals from humans (27; 40.9%), followed by cat (21; 31.8%) and opossum (12; 18.2%). The majority of farm blood meals were from horses (16; 66.7%), followed by human (2; 8.3%) and goat (2; 8.3%) (Figure 2.2).

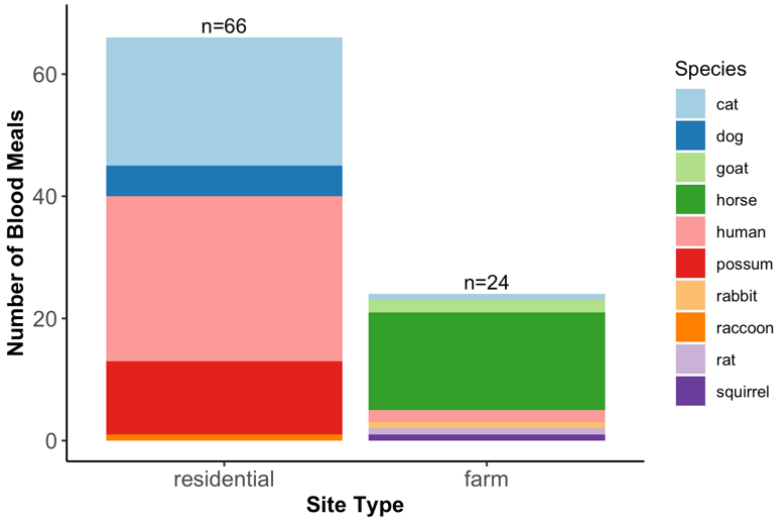

FIGURE 2.2: DISTRIBUTION OF BLOOD MEALS BY SITE TYPE

Distribution of *Ae. albopictus* blood meal source by sampling site: residential (n=4) and farm (n=4).

Host availability

Residential Host Feeding Index:

Household interview and blood meal data were used to calculate host feeding indices (HFIs), indicative of relative tendency to feed on certain vertebrate hosts across the residential properties where blood fed *Ae. albopictus* with identified blood meals were collected (n=28) (Table 2.2). The mean number (± SE) of blood meals from a given host type was calculated per residential property sampled: the most human blood meals were collected per residential property (0.96 ± 0.21), followed by cat (0.75 ± 0.17), and dog (0.18 ± 0.09). Similarly, there were the most human residents per residential property sampled (3.18 ± 0.36), followed by cat (0.39 ± 0.19), and dog (0.29 ± 0.10) according to household interviews. However, cats were reported to spend the most time outside over the 2 days prior to collection per residential property sampled (278.74 ± 232.93 min), followed by humans (234.26 ± 49.83 min), and dogs (53.61 ± 22.05 min) (Figure 2.3). The standard error in cat time was large because some properties had outdoor cats (24 hrs/d) while others did not have cats or had indoor cats.

TABLE 2.2: MEAN (± SE) NUMBER OF BLOOD MEALS, RESIDENTS, AND TIME SPENT OUTSIDE FOR HUMANS, CATS AND DOGS PER RESIDENTIAL PROPERTY SAMPLED

	Mean (± SE) per property		
Host (N)*	Blood meal/ property	Residents/ property	Time spent outside (min)/ property†
Human (27)	0.96 (0.21)	3.18 (0.36)	234.26 (49.83)
Cat (21)	0.75 (0.17)	0.39 (0.19)	278.74 (232.93)

Dog (5)	0.18 (0.09)	0.29 (0.10)	53.61 (22.05)

* N = total number of blood meals identified to host type from residential sites

† Mean (± SE) of within household sum of time spent outside by all residents of a given host type according to self-reported household interview data

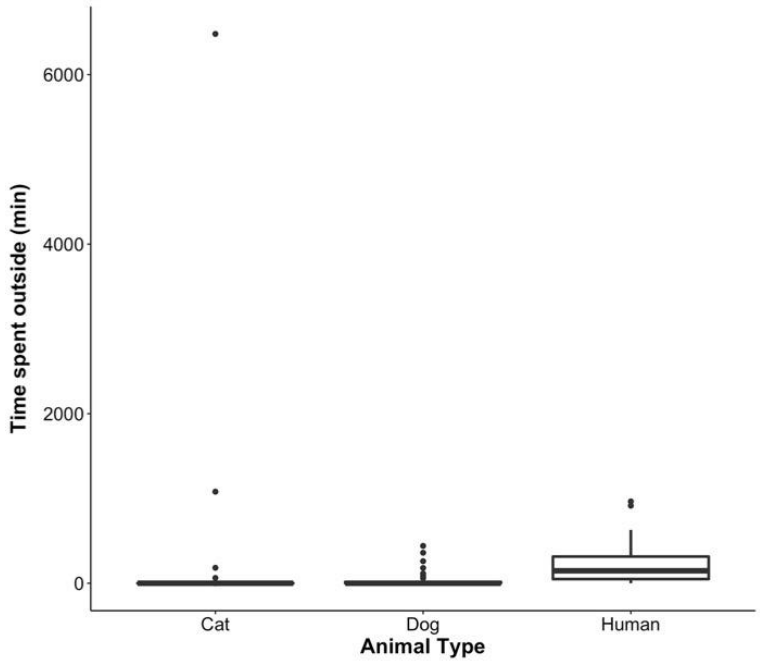

FIGURE 2.3: TIME OUTSIDE

Box plot of average time spent outside (min) by each animal type per residential property (n=28).

Mean numbers of blood meals and residents were used to calculate paired comparisons of feeding between humans, cats, and dogs through abundance and time-weighted HFIs (Table 2.3). Human vs cat HFI and HFI_T both demonstrated a tendency to feed on cats compared with humans (0.16 and 0.20). Likewise, human vs dog HFI and HFI_T both suggest that *Ae. albopictus* fed disproportionately often on dogs compared with humans (0.49 and 0.14). However, cat vs dog HFI and HFI_T produced opposite results: according to abundance measures, cats were fed

upon disproportionately more often compared with dogs (3.05), but when time-weighted, dogs were fed upon disproportionately more often compared with cats (0.73). On average, cats spent much more time outside than dogs, causing the directionality change of the index. Furthermore, neither HFI metric demonstrates a particularly strong deviance from the expected feeding proportions, suggesting that *Ae. albopictus* may not have a strong preference between cats and dogs.

TABLE 2.3: ABUNDANCE AND TIME-WEIGHTED HOST FEEDING INDICES FOR BLOOD FED *AE. ALBOPICTUS* COLLECTED ON RESIDENTIAL PROPERTIES

Index	Human vs Cat	Human vs Dog	Cat vs Dog
HFI*	0.16	0.49	3.05
HFI_T†	0.20	0.14	0.73

*Calculated with mean number of blood meals and residents per residential property sampled

† Calculated with mean number of blood meals, residents and mean time spent outside per residential property sampled

Residential Forage Ratio:

Forage ratios (FRs) were calculated from camera trap data at the 4 residential sites for all animals for which camera trap images were taken or blood meals identified (Table 2.4). Cats and opossums were fed upon more often than expected given their relative abundance in the host population. Of all residential blood meals taken from free roaming animals (i.e., not humans and dogs, which are largely constrained by property fences in residential sites sampled), 65.7 ± 10.2% were derived from cats, but only 27.4 ± 10.9% of all images were taken of cats, resulting in a 3.56 ± 0.98 FR (above the FR=1 threshold to infer preference). Opossum blood meals

accounted for 31.8 ± 10.8% of all blood meals but no opossums were pictured, resulting in an undefined FR, but suggesting preference for opossums. Raccoons, the other nocturnal animal detected by camera traps, were pictured often (24.8 ± 16.4% of all animals pictured) but only represented 2.5 ± 2.5% of all blood meals, resulting in a FR below 1 (0.046 ± 0.046), suggesting avoidance. Squirrels and birds were also pictured often (21.6 ± 10.5% and 26.2 ± 11.2% of all animals pictured, respectively) but no blood meals were identified from these host types in the blood fed *Ae. albopictus* collected at residential sites, resulting in a FR of 0, suggesting avoidance.

TABLE 2.4: MEAN (± SE) PERCENTAGE OF BLOOD MEALS, PERCENTAGE OF CAMERA TRAP IMAGES, AND FORAGE RATIO FOR ALL ANIMAL TYPES FOR WHICH CAMERA TRAP IMAGES WERE TAKEN OR BLOOD MEALS IDENTIFIED AT RESIDENTIAL SITES (N=4) IN SUFFOLK COUNTY, NY.

	Mean (± SE)		
	% of blood meals	% of images	Forage Ratio
Cat	65.7 (10.2)	27.3 (10.9)	3.6 (1.0)
Possum	31.8 (10.8)	0 (0)	∞*
Raccoon	2.5 (2.5)	24.8 (16.4)	0.05 (0.05)
Squirrel	0 (0)	21.6 (10.5)	0 (0)
Bird	0 (0)	26.2 (11.2)	0 (0)

*FR was infinite because division by zero is undefined

Farm Host Availability:

Approximate numbers and time spent outside for humans and domestic animals were reported by the farm owners. At Farm A, approximately nine people spent time at the farm for a total of 52

hours per day. The farm also had 40 horses, spending a total of 70 hrs/d outside. At Farm A, 3.6% of camera trap images were of cats, 67.9% of raccoons, 17.9% of foxes, 3.6% of deer, and 7.1% of squirrels. Blood meals collected at Farm A included 6 horse and 1 squirrel.

Farm B estimated that 30 people (180 hrs), 100 horses (200 hrs), 2 dogs (26 hrs), and 2 goats (26 hrs) were outside on the property per day. Of all camera trap images at Farm B, 37.1% were of cats, 44.3% of raccoons, 4.1% of opossums, 5.2% of deer, 5.2% of squirrels, and 4.1% of rabbits. The blood meals consisted of 5 horses, 1 human, and 1 rabbit.

Farm C estimated that 7 people (11 hrs), 46 horses (420 hrs), 2 dogs (12 hrs), 18 chickens (171 hrs), 4 ducks (38 hrs), and 1 goose (24 hrs) spent time outside per day. The most images were taken of cats (48.8%), followed by birds (23.3%), raccoons (14.0%), squirrels (9.3%) and rabbits (4.7%). Blood meals included 4 horses and 1 cat.

Farm D estimated that 3 people (14 hrs), 8 horses (48 hrs), 2 dogs (8 hrs), 20 goats (260 hrs), 4 sheep (52 hrs), 1 alpaca (24 hrs), 1 llama (24 hrs), 20 rabbits (260 hrs), 9 ducks (117 hrs), and 30 chickens (720 hrs) spent time outside per day. The camera trap pictured raccoons (33.3%) and birds (66.7%). Blood meals collected included: 2 goat, 1 horse, 1 human, and 1 rat.

Despite the diversity of hosts available at the 4 farm sites, the predominant blood meal identified at three of these sites was horse. The fourth farm was an anomaly, with more blood meals collected from goats than horses, but it was also the only farm where more goats were available than horses. Once again, raccoons were pictured at all sites, but no blood meals were collected, further suggesting avoidance of this animal. Birds were pictured frequently at 2 sites, and no blood meals collected, also suggesting avoidance.

Fitness by Host Blood Source

Within-population fitness impacts for NY *Aedes albopictus*: Table 2.5 presents the proportions of *Ae. albopictus* that laid and retained mature eggs and mean (± SE) number of eggs produced by blood source.

TABLE 2.5: EGG PRODUCTION BY BLOOD MEAL SOURCE FOR NY *AE. ALBOPICTUS*

Blood Source	Proportion which laid eggs (%)	Proportion with retained eggs (%)*	Mean eggs produced (± SE)†
Human	104/121 (86.0)	23/121 (19.0)	61.0 (2.9)[a]
Opossum	64/86 (74.4)	10/86 (11.6)	58.7 (4.8)[a]
Rat	100/122 (82.0)	16/122 (13.1)	53.5 (3.7)[ab]
Horse	70/97 (72.2)	11/97 (11.3)	48.5 (3.9)[ab]
Cat	57/89 (64.0)	10/89 (11.2)	40.3 (4.0)[b]

* Includes mosquitoes with any number of retained eggs

† Groups that do not share a superscript letter are significantly different.

Females that ingested cat blood resulted in lower fecundity compared with those fed human and opossum blood (β= -17.3, SE=5.3, *P*=0.01 and β= -20.9, SE=5.9, *P*=0.004, respectively). There was no significant difference between any other blood group (Figure 2.4a). There was also no significant effect of survival time on number of eggs produced (although only one blood meal was provided in this study, which may limit impact of extended survival). On average, female *Ae. albopictus* began laying on day 3 post-blood meal, regardless of blood meal source, and survived for 7-9 days. Notably, there were significant differences between replicates ($p < 0.0001$).

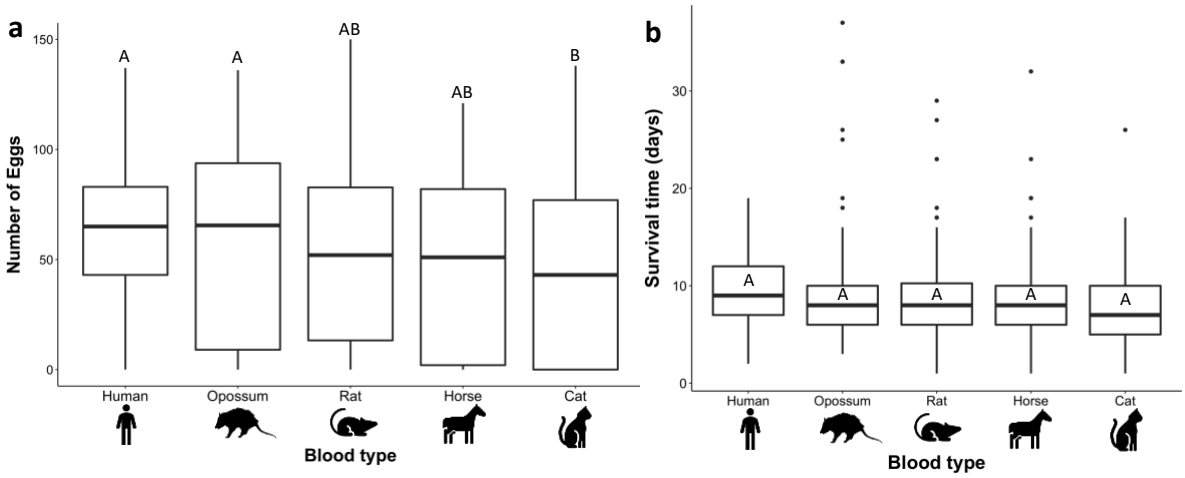

FIGURE 2.4: FECUNDITY AND SURVIVAL OF NY *AE. ALBOPICTUS*

Box plots for NY *Ae. albopictus* female mosquitoes for **a)** number of eggs produced and **b)** survival time in days when fed cat (n=89 females for egg production and n=90 females for survival), horse (n=97 egg production; n=98 survival), human (n=121 egg production; n=123 survival), rat (n=122 egg production; n=124 survival), and opossum blood (n=86 egg production; n=92 survival). Groups that do not share a letter are significantly different.

There were no significant differences in *Ae. albopictus* female survival time between any of the host blood groups (Figure 2.4b). Mosquitoes fed human blood survived 9.6 (±0.3) days, opossum-fed survived 9.5 (±0.6) days, rat-fed survived 8.7 (±0.4) days, horse-fed survived 8.6 (±0.48) days, and cat-fed survived 7.6 (±0.45) days. There were significant differences in survival by replicate ($p<0.0001$). Daily survival curves averaged over replicates performed for each host type are presented in Figure 2.5.

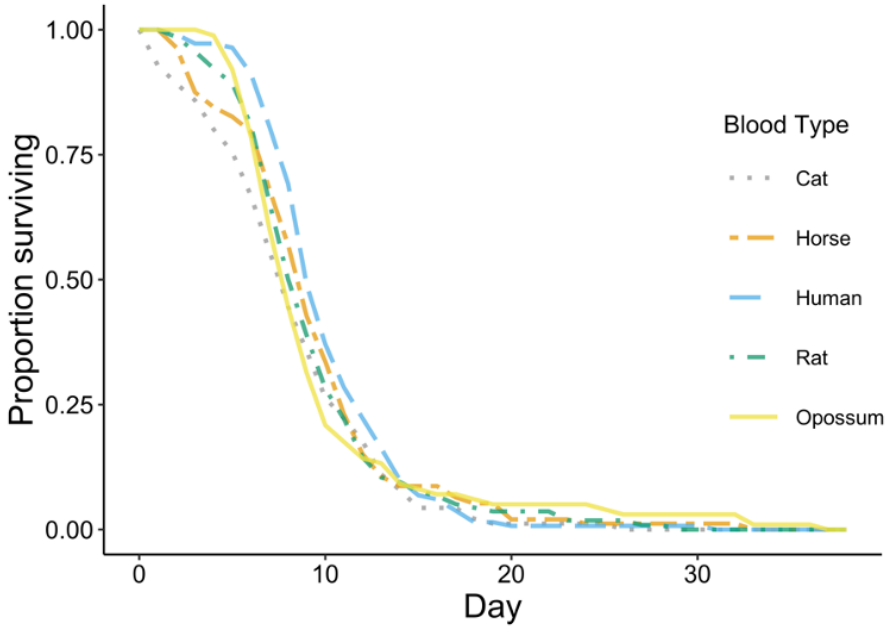

FIGURE 2.5: NY *AE. ALBOPICTUS* SURVIVAL CURVES

Survival (days) of NY *Ae. albopictus* by host blood type ingested, including cat (n=90 females), horse (n=98), human (n=123), rat (n=124), and opossum (n=92) blood.

The mean (±SE) R_0 across replicates was 29.7 (±4.1) for *Ae. albopictus* fed human blood, 27.1 (±8.9) for opossum blood, 27.0 (±4.1) for rat, 22.9 (±5.7) for horse, and 19.5 (±6.5) for cat. No significant differences in (R_0) were found by host blood group.

Between-population differences of NY and Baltimore *Ae. albopictus*: The proportions of *Ae. albopictus* that laid and retained mature eggs, mean (± SE) eggs, and mean (± SE) eggs/mm wing length is reported in Table 2.6. Wing lengths were measured for 26-33 females per colony per replicate.

TABLE 2.6:EGG PRODUCTION FOR NY AND BALTIMORE *AEDES ALBOPICTUS* FEMALES FED HUMAN OR RAT BLOOD

Origin and Blood Source	Proportion which laid eggs (%)	Proportion with retained eggs (%)	Mean eggs produced (± SE)	Mean eggs/mm wl produced (± SE)*
NY Human	76/89 (85.4)	17/89 (19.1)	58.8 (3.6)	20.7 (1.3)[a]
NY Rat	75/95 (78.9)	13/95 (13.7)	46.1 (4.0)	16.2 (1.4)[b]

Baltimore Human	73/89 (82.0)	12/89 (13.5)	41.4 (3.2)	14.7 (1.1)[b]
Baltimore Rat	70/95 (73.7)	11/95 (11.6)	38.2 (3.5)	13.6 (1.3)[b]

*Groups that do not share a superscript letter are significantly different.

The only significant differences in number of eggs produced per mm wing length were between NY mosquitoes fed human blood and the three other host blood source groups (Figure 2.6a). Baltimore mosquitoes fed human (β= -6.0, SE=1.8, *P*=0.0008) and rat blood (β= -6.9, SE=1.8, *P*=0.0001) produced fewer eggs/mm wl than NY mosquitoes fed human blood. NY mosquitoes fed human blood produced more eggs per mm wl than those fed rat blood (β= 3.8, SE=1.8, *P*=0.03). Baltimore mosquitoes fed rat blood produced marginally fewer eggs/mm wl than NY mosquitoes fed rat blood (β= -3.1, SE=1.7, *P*=0.07). There was no significant difference in eggs produced/mm wl between Baltimore mosquitoes fed human and rat blood (β= 1.0, SE=1.8, *P*=0.6) or Baltimore mosquitoes fed human blood and NY mosquitoes fed rat blood (β= -2.1, SE=1.8, *P*=0.2). There were significant differences in between replicates ($p < 0.0001$).

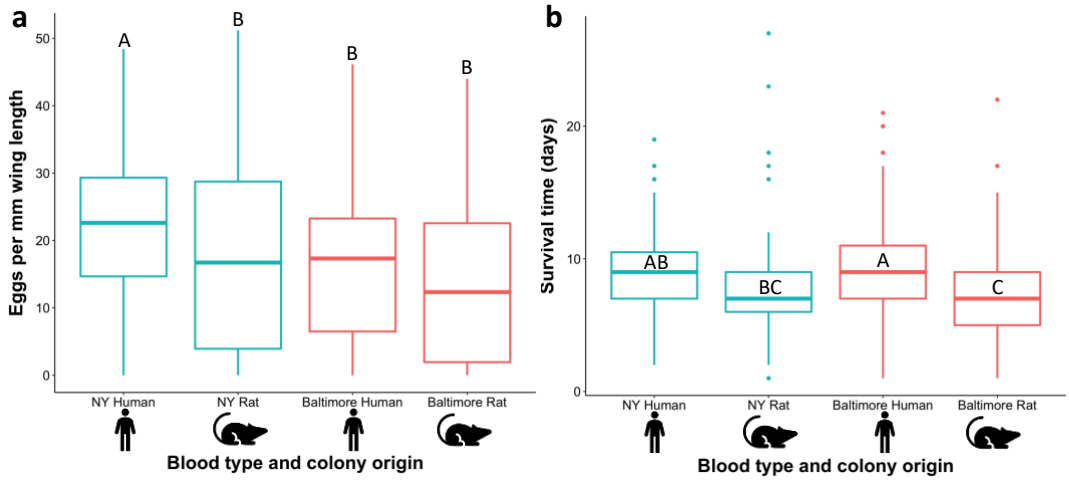

FIGURE 2.6: NY AND BALTIMORE *AE. ALBOPICTUS* FECUNDITY AND SURVIVAL

Box plots for Baltimore and NY *Ae. albopictus* female mosquitoes of **a)** number of eggs produced when fed rat and human blood; and **b)** survival in days. Sample sizes for Baltimore human and rat and NY human and rat were, respectively: n=89, 95, 89, and 95 for egg production and n=94, 95, 91, and 97 for survival. Groups that do not share a common letter are significantly different.

The mean (± SE) survival time of Baltimore *Ae. albopictus* was significantly higher when fed human blood (9.6 days ±0.4) compared with rat blood (7.2 days ±0.4) (β= 2.3, SE=0.5, *P*=0.0001). The same survival trend was observed for NY *Ae. albopictus* where mosquitoes fed human blood survived marginally longer than those fed rat blood (9.0 days ±0.3 and 7.7 days ±0.4 respectively: β= 1.3, SE=0.5, *P*=0.08) (Figure 2.6b). Baltimore mosquitoes fed human blood survived significantly longer compared with NY mosquitoes fed rat blood (β=1.9, SE=0.5, *P*=0.002). Survival time was significantly lower for Baltimore mosquitoes fed rat blood compared with NY mosquitoes fed human blood (β=-1.7, SE=0.5, *P*=0.008). There was no significant difference in survival time between mosquitoes fed human blood from both sites (β= 0.6, SE=0.5, *P*=0.6) or fed rat blood from both sites (β= -0.4, SE=0.5, *P*=0.8). We detected differences by replicate (p=0.007). Daily survival curves averaged over the three replicates are presented in Figure 2.7.

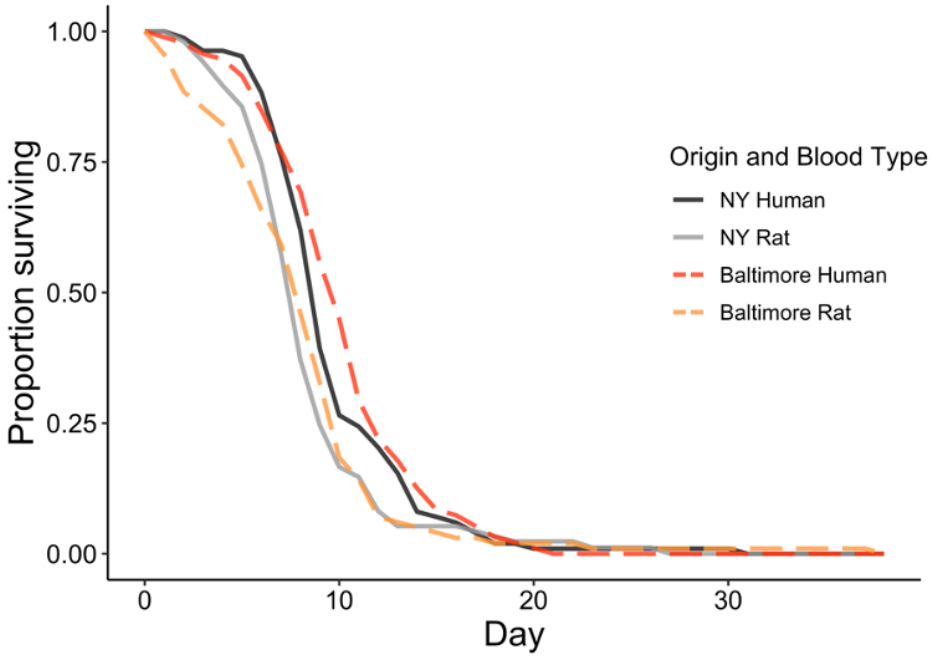

FIGURE 2.7: NY AND BALTIMORE *AE. ALBOPICTUS* SURVIVAL CURVES

Survival (days) of Baltimore and NY *Ae. albopictus* fed human (n=94 and 91, respectively) and rat (n=95 and 97, respectively) blood. Curves are averaged over three replicates.

The mean (±SE) basic reproductive rate (R_0) (averaged over 3 replicates) of Baltimore *Ae. albopictus* fed human blood was 20.4 (±1.2), 19.7 (± 4.6) for Baltimore rat, 29.3 (± 5.7) for NY human, and 24.5 (± 4.5) for NY rat. No significant differences were found for R_0 among any of the blood/colony combinations.

Discussion

Mosquito feeding behavior plays a vital role in disease transmission, however, it can be difficult to quantify and predict because there are diverse factors that influence feeding behavior in nature. We investigated the feeding patterns of the globally invasive vector, *Ae. albopictus*, from eight sampling sites, categorized as farm and residential habitats at the northern edge of its range

in the United States. In tandem, we addressed two factors that may influence these patterns: host availability and variation in mosquito fitness from different host blood sources. We detected ten host species, some of which were over- or under-utilized compared with their availability as measured by host feeding indices and forage ratios. Host blood source had a limited impact on mosquito survival, egg production, and basic reproductive rate, indicating that fitness does not play a significant role in predicting *Ae. albopictus* feeding patterns in the Northeastern US.

The ten host species we detected in *Ae. albopictus* blood meals from Long Island, NY are hosts previously reported for *Ae. albopictus* elsewhere in the world. The proportion of human blood meals (32.2%) identified in Long Island was lower than reported in many other locations worldwide[5, 6, 12-17, 19-23], but was higher than in some other studies from the United States (Hawaii, Missouri, North Carolina, Maryland, and Virginia)[7-11]. More *Ae. albopictus* fed on cats in our study on Long Island than in any other location previously reported, with the exception of Virginia [9]. The third most common host for this mosquito species on Long Island, the horse, has only been detected in four of eighteen previous *Ae. albopictus* blood meal studies and at lower levels[8, 11, 13, 15]. Similarly, the fourth most common host, opossum, has been reported in five previous studies, also at lower levels, with the exception of Virginia[9-11, 14, 16]. Long Island *Aedes albopictus* fed less frequently on dogs compared with the representative proportion in numerous other studies[8, 10, 11, 14-17]. Notably absent from the Long Island blood meals were cows, deer, and birds, all of which were present on at least one site in our study and have been detected in at least six previous blood meal studies. It is possible that a larger sampling of blood meals may have revealed these hosts, however, birds have also been absent from most other studies in Northeastern USA[7, 14, 16]. Notably, only about half of collected blood meals were successfully identified to species, but the reason for the low success rate is unknown. It is possible that this

may have biased the species that were identified, however, tests of primer versatility performed by Reeves et al. (2018) showed amplification for the majority of vertebrate species (90/93)[36].

This is only the third study of *Ae. albopictus* blood feeding biology that quantitatively assessed host availability, and the first to do so with wild animals. Abundance and time-weighted host feeding indices (HFIs) calculated using household interview data revealed disproportionately high levels of feeding on cats and dogs compared with humans. Richards et al. (2006) reported a similar trend for HFIs based on host abundance in North Carolina, but when time-weighted, found that humans were fed upon disproportionately often compared with cats and dogs[11]. In Brazil, HFIs based on host abundance showed the opposite trend to ours, suggesting that *Ae. albopictus* fed disproportionately often on humans compared with cats and dogs[13]. These results highlight the need for additional studies that measure host availability and also suggest a need for caution when extrapolating these results to make conclusions about innate mosquito preference. In both Long Island and North Carolina, collections were only conducted at a subset of houses per neighborhood, allowing for the movement of blood fed mosquitoes from properties where interviews were not conducted. Flight range for engorged blood fed *Ae. albopictus* is not known, but it is likely that movement between properties is possible after feeding according to the reported range of other blood fed species and records of *Ae. albopictus* dispersal between blood feeding and oviposition[47-50]. Furthermore, household interview data depend on accurate self-reporting of outdoor activity, which may be unreliable[51]. This inaccuracy of outdoor time estimates is compounded if the interview is only administered once for the entire sampling period, such as in Richards et al. (2006)[11]. Another potential source of bias is insecticide/endectocide use for domesticated animals[52] – we did not gather data on this and

therefore cannot determine whether this may have impacted observed feeding patterns by limiting domestic animal blood meals.

We also assessed host availability through camera traps in order to calculate forage ratios for free-roaming animals, which suggested a tendency to feed on cats and opossums and to avoid raccoons, squirrels, and birds compared with their relative abundance in residential sites. While camera traps do not provide a perfect measure of host abundance, it is considered a robust method for mammal inventories[53]. Camera traps may be less useful in estimating bird abundance[54], however, birds were one of the most frequently photographed groups of animals in our study, but were not fed upon, so improved accuracy in estimating bird abundance would not have altered conclusions drawn from forage ratio calculations. Forage ratio calculations were limited to animals that tend to cross freely between yards despite fences, including all wild animals and cats, but excluding humans and dogs. Camera traps were only placed in 2 properties per site, limiting the utility of camera traps to assess the site-wide availability of these animals with high property-line fidelity. Furthermore, camera traps were not operated for the full collection period – twenty blood meals were collected prior to camera trap deployment. Host availability can shift over the season[55], so this may have impacted our results.

For both household interviews and camera trap host census methods, heterogeneity in host availability between sampled households can lead to uneven exposure of mosquitoes to a given host. When analyses are conducted across many households, as in this study, this heterogeneity can be lost. The level at which host availability measures and bloodmeals are grouped can impact the interpretation[11]. This may be particularly relevant when considering hosts with a high level of variation in time spent outdoors, such as cats in this study.

Despite limitations, estimating host availability and abundance in conjunction with blood meal studies is much more informative than studies that lack such data. By understanding more about the context in which a certain feeding pattern arose, more general conclusions can be drawn about feeding behavior. However, the patterns revealed after accounting for host availability can be caused by numerous factors, such as host defenses. This may explain the high number of opossum blood meals because this nocturnal marsupial would likely be asleep, with decreased self-defense, during *Ae. albopictus* daytime biting activity. However, raccoons are also nocturnal and in contrast, were fed upon less often than expected, suggesting that innate preferences or other factors could potentially also be at play. Only two preference studies have been conducted for *Ae. albopictus*; in La Reunion Island, a no-choice blood feeding experiment on 12 host types found chicken, human, dog and cow were fed upon more often than duck, shrew, rat, pig, mouse, goat, gecko, and chameleon[25]. Subsequently, a choice experiment showed higher attraction to humans than to chicken, dog, cow and goat[25]. However, large and small animals were treated differently and were not given equal opportunities for self-defense, potentially affecting results. In Thailand, landing catches demonstrated preference for humans compared to pigs, buffalo, dogs, and chickens; however, the use of a second human to catch mosquitoes from the non-human animals may have impacted results. It therefore remains unclear whether *Ae. albopictus* has innate host preference.

One mechanism by which host preferences may evolve is through natural selection whereby feeding on a certain host enhances reproductive fitness, leading selection to favor genetic variants with preference for that host. This is known to be the case for other species, such as *Ae. aegypti*[4, 28]. We investigated the potential role of fitness in driving *Ae. albopictus* feeding patterns by assessing survival and egg production of mosquitoes after feeding on blood from

several host blood sources in the Northeastern United States. Within the NY *Ae. albopictus* population, we found that host blood source had very limited impact on survival, egg production, or basic reproductive rate. The only significant differences were lower egg production after feeding on cats compared with humans and opossums, and no significant differences in survival. Interestingly, the reduced fecundity on cat blood is opposite to what we might expect based on the feeding index, which suggested a tendency to feed more often on cats compared with humans. There are many reasons that these contrasting results may have occurred, including possible under-estimation of cat availability in host censuses leading to an inflated HFI, potentially lower levels of host defenses among cats compared with other animals leading to higher feeding success rate, and the possible evolution of preference via selection on other traits. Additionally, eggs used to establish the NY colony included some sites with a wider geographic spread (~78 km) than that studied for feeding patterns in the field (~40 km). This broader geographic origin may have impacted the results if variability for this trait exists within southern NY. This may have obscured more location-specific effects of blood type if they existed.

A previous report from Baltimore of high feeding rates on rats, led us to compare the fitness of NY and Baltimore *Ae. albopictus* after feeding on human and rat blood. Specifically, we investigated whether differences in fitness may be driving the striking differences in feeding patterns between the two locations. However, the only significant difference was higher egg production by NY mosquitoes fed human blood than all three other groups. If egg production was driving this difference, we would expect to also see higher egg production for Baltimore mosquitoes fed rat compared with human blood, but this was not the case. Furthermore, survival of mosquitoes fed on human blood was longer than those fed on rat blood for both Baltimore and NY *Ae. albopictus.* Together, these results suggest that a fitness advantage does not drive

different feeding patterns in these two locations. The authors of the Baltimore study did not quantitatively assess host availability; however, they suggest that the percentage of abandoned properties and time spent in by residents in backyards (unpublished data) varied by neighborhood and corresponded with human blood meal proportion[7]. In the absence of detected fitness benefits, it is possible that host availability was the driver of feeding pattern differences.

The impact of host blood source on *Ae. albopictus* egg production has only been assessed twice before. Gubler (1970) found greater fecundity for mouse-fed females, followed by guinea pig, rat, and chicken; however, the study was not replicated and no statistical analyses were conducted[30]. In another study, chicken-fed *Ae. albopictus* were less fecund than those offered guinea pig or human blood and, consistent with our results, no differences between the two mammals were found[29]. These results do not demonstrate a selective pressure for *Ae. albopictus* to evolve preferences within mammalian hosts. However, preference can evolve through other pathways and should be assessed directly. Other specialist feeders lack apparent fitness advantages for their preferred host. For example, *Anopheles gambiae* has a well-established preference for humans, but in a single study conducted to date, there is no fitness advantage provided by a human-only diet compared with a generalist diet[56].

It is also possible that when assessed under different conditions, differences in fitness by host blood source may be revealed. For instance, we did not provide the mosquitoes with sugar after blood feeding; the presence of sugar has been shown to reduce reproductive fitness in *Ae. albopictus* compared with human blood alone and mosquitoes on Long Island feed frequently on sugar[42, 57]. For *Ae. aegypti,* the addition of sugar changed the directionality of host blood source effects on fitness, shifting the fitness benefits from human to mouse blood[28]. If a similar phenomenon exists for *Ae. albopictus*, the absence of sugar in our experiments would maximize

the fitness of human blood compared with other host types. We also only provided the mosquitoes with one blood meal. Providing a series of blood meals may have influenced our results[29].

Aedes albopictus is often referred to as anthropophilic due to the high percentage of human blood meals in numerous field studies and the preference assessments conducted by Delatte et al. (2010)[25]. However, this classification remains unproven. In fact, our results are more indicative of a generally mammalophilic feeding behavior for *Ae. albopictus*. It is important to understand the underlying blood feeding behavior and physiology of *Ae. albopictus* because it influences and modulates the feeding patterns in the field, which will ultimately influence pathogen transmission[24]. In Long Island, the diverse utilization of hosts in residential and farm settings demonstrates that *Ae. albopictus* could serve as an enzootic bridge vector. However, the absence of bird blood meals suggests that *Ae. albopictus* may be of limited concern as a vector of West Nile and Eastern equine encephalitis viruses in the Northeastern US. Populations of *Ae. albopictus* in this region have sufficient vector competence to transmit numerous anthroponotic viruses[58-60], but transmission of these pathogens may be limited due to lower rates of human feeding compared with other regions[61].

Our results provide insight into blood feeding hosts which may influence disease transmission risk by *Ae. albopictus* in Northeastern United States. Additionally, our observations reveal that integrating host availability measures into mosquito blood meal studies is important to interpret feeding patterns, but does not fully explain blood meal distribution. Fitness benefits did not explain the feeding patterns observed in NY or Baltimore, highlighting the need for further research on determinants of *Ae. albopictus* feeding behavior.

Data Availability: Data availability: Data will be deposited in Cornell University Library's institutional repository, eCommons (https://ecommons.cornell.edu), for preservation and access. Datasets will be available via the world wide web without restriction at this DOI: https://doi.org/10.7298/84ky-sv64. eCommons provides each item with a persistent identifier and is committed to preserving the binary form of the digital object.

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Supplemental Material 1: Household interview

1. Address: _____
2. How many people were living in this house over the past 2 days?_____
3. Were any animals staying here over the past 2 days? _____
4. (if yes) What type of animal? How many? _____
5. We are counting total human hours outside the house in the yard. How much time have people spent outside in your yard today, added all together?_____ (If prompting is needed, say "For example, if 2 people spent one hour outside so far today, that would be 2 hours")
6. How much time did people spend outside in your yard yesterday? _____
7. How much time did people spend outside in your yard 2 days ago? _____
8. How much time did your pet(s) spend outside in your yard today? _____ Yesterday? _____ 2 days ago?_____
   ****_Separate by type of animal; ie collect info separately for cats and dogs but add together time for 2 dogs._
9. Did people wear mosquito repellent when they went outside over the past 2 days? (If yes, prompt "Always, usually or sometimes?"
   ☐ Always ☐ Usually ☐ Sometimes ☐ Rarely ☐ Never
10. Did you do anything else to avoid mosquito bites, such as use insecticides during the last week? _____
    _____

Were windows open? Y / N Did they have screens? Y / N

Were doors open? Y / N Did they have screen doors? Y / N

Supplemental Material S2. New York *Aedes albopictus* colony details

Eggs were collected in the field in 2019 for another study by Talya Shragai (Shragai 2020). Mosquitoes were reared in separate colonies by location for a few generations and then combined into one NY colony.

The mosquitoes utilized in the life table had been in laboratory colony (sum of generations passed in location-specific and general NY colonies) for between 6 (replicate 1) and 10 (replicate 4) generations.

Eggs were collected from the following sites:

County	**Site Name**
Westchester	Yonkers
Rockland	Spring Valley
Suffolk	Babylon
Suffolk	Central Islip
Suffolk	Smithtown

The Babylon and Central Islip sites were the same as those sampled in our study. Smithtown is also on Long Island and Yonkers and Spring Valley are in the Hudson Valley, within 15 km of Long Island. The Spring Valley site is approximately 78 km from the furthest East site in our study. The distance between the furthest west and furthest east blood meal collection sites in our study is 40 km. Therefore, this colony encompasses mosquitoes from a larger geographic area in NY compared with the blood meal analysis collection sites.

Chapter 3 : *Aedes albopictus* host odor preference does not drive observed variation in feeding patterns across field populations[*]

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Abstract: Laboratory and field-based studies of the invasive mosquito *Aedes albopictus* demonstrate its competency to transmit over twenty different pathogens linked to a broad range of vertebrate hosts. The vectorial capacity of *Ae. albopictus* to transmit these pathogens remains unclear, partly due to knowledge gaps regarding its feeding behavior. Blood meal analyses from field-captured specimens have shown vastly different feeding patterns, with a wide range of anthropophagy (human feeding) and host diversity. To address this knowledge gap, we asked whether differences in innate host preference may drive observed variation in *Ae. albopictus* feeding patterns in nature. Low generation colonies (F2-F4) were established with field-collected mosquitoes from three populations with high reported anthropophagy (Thailand, Cameroon, and Florida, USA) and three populations in the United States with low reported anthropophagy (New York, Maryland, and Virginia). The preference of these *Ae. albopictus* colonies for human *versus* non-human animal odor was assessed in a dual-port olfactometer along with control *Ae. aegypti* colonies already known to show divergent behavior in this assay. All *Ae. albopictus* colonies were less likely ($p<0.05$) to choose the human-baited port than the anthropophilic *Ae. aegypti* control, instead behaving similarly to zoophilic *Ae. aegypti*. Our results suggest that variation in

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reported *Ae. albopictus* feeding patterns are not driven by differences in innate host preference, but may result from differences in host availability. This work is the first to compare *Ae. albopictus* and *Ae. aegypti* host preference directly and provides insight into differential vectorial capacity and human feeding risk.

Introduction: Mosquito blood feeding behavior is a critical determinant of pathogen transmission. Some species have innate host preferences, actively choosing to feed on one host species or class over others[1-3]. Other mosquito species are host generalists, and exhibit little to no preference for particular host species or groups. Host preference plays a role in host contact rates, interacting with external factors, such as host availability, to influence feeding patterns in the field[1,4,5]. Feeding patterns, in turn, influence the probability of mosquito contact with infectious host reservoirs and onward transmission to susceptible hosts[6,7].

Despite the important role of host preference in pathogen transmission, this trait is not well characterized for the tiger mosquito, *Aedes albopictus*, a highly invasive nuisance species with the potential to transmit over twenty pathogens that infect a range of vertebrate host species[8,9]. Several of these pathogens share another mosquito vector, *Ae. aegypti*, including dengue, Zika, and chikungunya viruses. In contrast to *Ae. albopictus*, *Ae. aegypti* host preference is well characterized. In its invasive range outside of Africa, *Ae. aegypti* is strongly anthropophilic, preferring the odor of humans over that of other host species[10,11]. Within its ancestral range in Africa, *Ae. aegypti* is more diverse and exhibits a range of host preferences, from zoophilic (non-human preferring) to anthropophilic (human preferring)[11-13]. Human-preferring populations in and out of Africa are genetically related, sharing common mutations in

several chromosomal regions[11], which provides additional support for the genetic underpinnings of human preference[13].

Aedes albopictus host preference has only been assessed twice to date, despite its importance as a major vector and nuisance species. In Thailand, landing catches were performed, comparing attraction of wild mosquitoes to human, pig, buffalo, dog, and chicken[14]. In La Réunion, preference assays were conducted with human, cow, dog, goat, and chicken, measuring choice between human and each of the non-human animals by releasing mosquitoes in an enclosure and subsequently assessing host feeding rates[15]. The results of both studies indicate a preference for humans over the other animals tested. However, the results from the La Réunion study may have been influenced by host defenses[15]. Human subjects may have avoided defending themselves since they knew they were in a scientific study while the non-human animals would likely have exhibited typical host defense behaviors that may have impacted feeding success[16]. Note, however, host defenses were not addressed in the text[15]. In La Réunion, no-choice (single host) assays were also conducted, measuring feeding on human, pig, goat, cow, dog, duck, chicken, rat, chameleon, gecko, mouse, and shrew in the absence of other hosts[15]. *Aedes albopictus* fed readily on chicken, human, dog, and cow, and significantly less often on all other hosts assessed.

While little is known about *Ae. albopictus* host preference, there has been robust investigation of its feeding patterns. Feeding patterns are distinct from host preference in that patterns describe mosquito-host associations in nature and are influenced by environmental and biological parameters, whereas preference describes the innate tendency of a mosquito species to choose a certain host over others[1]. *Aedes albopictus* feeding patterns have been assessed across nineteen blood meal analysis studies from across the world, the results of which exhibit a

remarkably diverse range of feeding (reviewed by Fikrig and Harrington, 2021[1]). The percent of blood meals identified as human ranged from 3.9-100% and the number of host species identified ranged from three to fifteen. The cause of this striking variability in feeding patterns is unknown. Methodological differences, such as blood meal analysis and collection techniques may explain some of the differences. There were also likely differences in host availability. However, only three of these studies quantified host availability[17-19], so it is impossible to retrospectively determine the extent to which external factors drove the differences in host usage. Another possibility is that *Ae. albopictus* populations vary in genetically-based host preference, similar to *Ae. aegypti* populations within Africa, thus driving divergent feeding patterns[11].

Aedes albopictus has high levels of phenotypic variation for numerous traits, including diapause[20], fecundity-size relationships[21], competitive interactions[21], larval growth rate[22], and viral susceptibility[23]. It also has a large genome[24] and substantial levels of genetic variation[25-27], although the level of variation among populations is different in different parts of the world [28]. *Aedes albopictus* genetic variation has been shown to underpin phenotypic variation of several traits, including vector competence[29] and diapause[30-32]. This trait variation has been credited for the impressive invasive potential of *Ae. albopictus* and its widespread establishment across a variety of climates and habitats[33,34].

It is unclear whether such variation exists for host preference among *Ae. albopictus* populations; the two host preference studies conducted to date do not provide insight into the possibility that innate host preferences vary in relation to observed differences in feeding patterns. The first *Ae. albopictus* host preference study referenced above was conducted in Thailand[14], where a blood meal analysis revealed that 100% of blood fed *Ae. albopictus* fed on humans, the most anthropophagic feeding pattern reported to date[35]. The other was conducted in

La Réunion, which was the site of a chikungunya epidemic transmitted by *Ae. albopictus*, suggesting a high level of human feeding (although no blood-feeding pattern study has been performed to corroborate this)[36,37]. There has been no experimental assessment of host preference from locations where *Ae. albopictus* populations have lower levels of anthropophagy (human feeding), nor any comparison of host preference between discrete populations.

We investigated whether population-level variation in *Ae. albopictus* host preference drives the divergent feeding patterns reported around the world. Using a dual-port olfactometer to simultaneously present human and guinea pig odors, we measured the host preference of low-generation *Ae. albopictus* colonies derived from six populations around the world: three from populations with previously reported low levels of anthropophagy in the United States (New York[17], Maryland[38], and Virginia[39]), and three from populations with high levels of anthropophagy (Florida, USA[40], Cameroon[41], and Thailand[35]). We directly compared these colonies to anthropophilic and zoophilic *Ae. aegypti* colonies, thus also providing the first direct comparison of the host preferences of these two vector species.

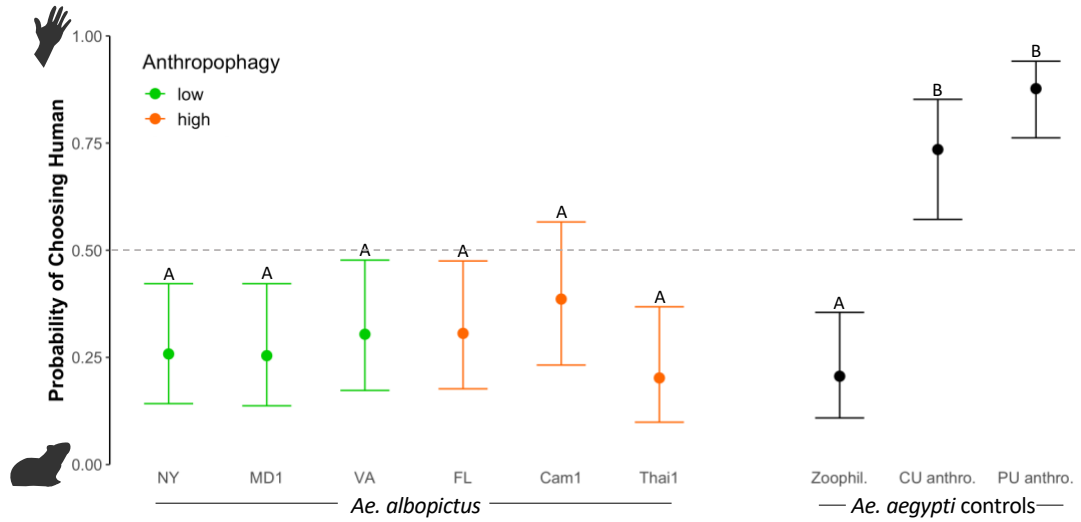
Results

Using a dual-port olfactometer, we measured the host preference of *Ae. albopictus* colonies derived from three anthropophagic and three zoophagic populations, as well as one zoophilic and two anthropophilic *Ae. aegypti* colonies. Eight replicates were conducted across two separate experimental rounds. Additionally, in the second experimental round, we assessed biological replicate colonies of three *Ae. albopictus* populations (established from a site at least 1.5km away from the primary colony collection site) and an anthropophilic *Ae. aegypti* transport control (to control for potential effects of transportation from the laboratory at Cornell to Princeton). For

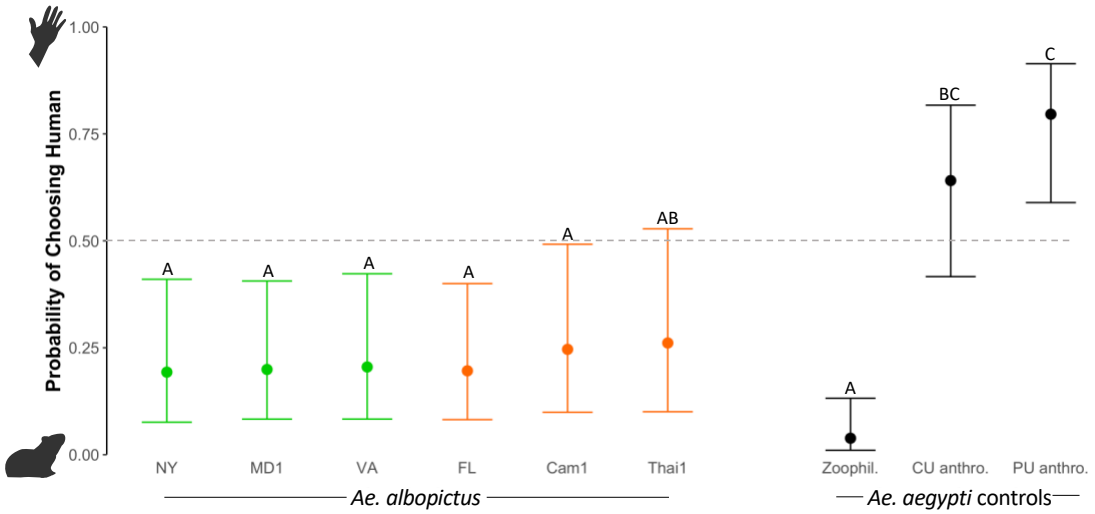
each of the colonies, we report the predicted probability of choosing human over guinea pig. We assessed whether differences in host choice existed between the colonies by analyzing the data together and for each experimental round separately.

For the combined analysis, all six *Ae. albopictus* colonies were more likely to choose guinea pig than human. The predicted probabilities of choosing human were below 0.5, including both the anthropophagic and zoophagic *Ae. albopictus* colonies (Fig. 3.1A, Supplemental table S3.1). As expected, the zoophilic *Ae. aegypti* colony was more likely to choose guinea pig than human and the two anthropophilic *Ae. aegypti* colonies were more likely to choose human than guinea pig. All *Ae. albopictus* colonies were clearly zoophilic, with the exception of Cameroon 1. This was the only *Ae. albopictus* colony with an upper confidence limit that crossed 0.5, the dividing line between whether a colony is more likely to choose human or guinea pig (predicted probability = 0.386, lower CL= 0.232, upper CL=0.566). Because this upper confidence limit was greater than 0.5, it is the only *Ae. albopictus* colony for which we cannot preclude the possibility of human preference under these experimental conditions, although the predicted probability was below 0.5. Notably, Cameroon 1, and all five other *Ae. albopictus* colonies were still significantly less likely to choose human than the two anthropophilic *Ae. aegypti* colonies (α=0.05) and did not behave significantly differently from the zoophilic *Ae. aegypti* (Supplemental table S3.1).

a. Combined Model



b. First Round Model



c. Second Round Model

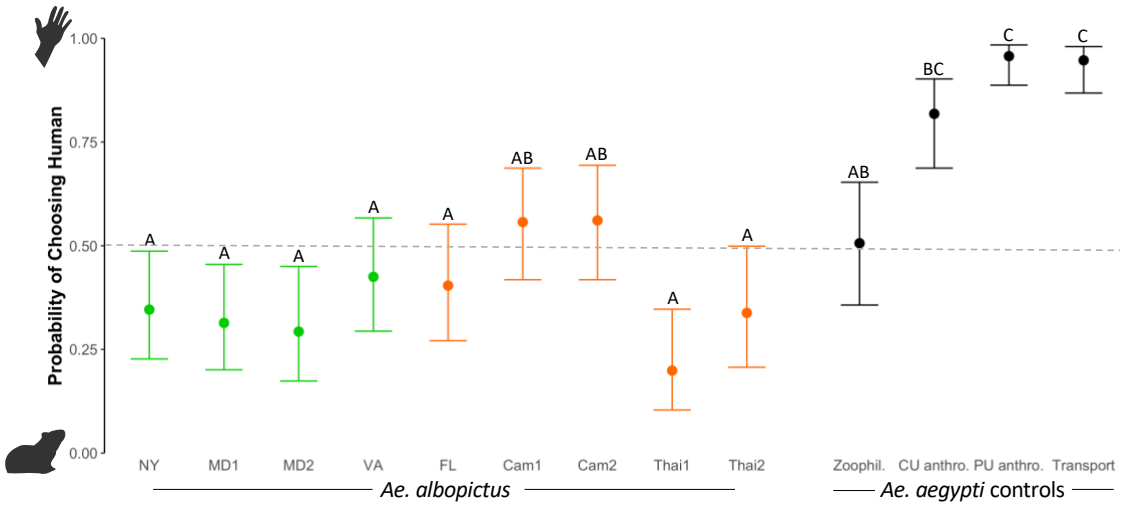


FIGURE 3.1: PREDICTED PROBABILITY OF CHOOSING HUMAN

This figure shows the predicted probability of choosing human for each of the colonies in: **a.** the combined model, **b.** the first round model, and **c.** the second round model. Within each graph, colonies that do not share a letter above the upper confidence limit are statistically different ($p<0.05$). The green colonies are *Ae. albopictus* derived from populations with previously reported low levels of anthropophagy, the orange colonies are *Ae. albopictus* from populations with previously reported high levels of anthropophagy, and the blue are *Ae. aegypti* control colonies. The dashed grey line indicates a 0.5 probability of choosing human, the level at which a colony would be equally likely to choose human or guinea pig; above this line, the colony is more likely to choose human and below, guinea pig. The first and second round trials were performed with minor methodological differences, including arm presentation. The following colony abbreviations were used: NY=New York, MD1= Maryland 1, VA=Virginia, FL=Florida, Cam1=Cameroon1, Thai1=Thailand 1, Zoophil.=Zoophilic, CU anthro.= Cornell anthropophilic, PU anthro.=Princeton anthropophilic.

We then examined the results for each round separately due to slight technical differences between the method of human host presentation (elbow versus forearm and hand). We also added a transport control and biological replicates for three *Ae. albopictus* colonies in the second round to control for potential effects of transportation from the laboratory at Cornell to Princeton (ca. 4-hour drive in a car with human odor) and potential founder effects, respectively. The first-round model included data from the four first-round replicates and was largely consistent with our analysis of both rounds combined with slight differences in the probability of choosing human over guinea pig (Fig. 3.1B, Supplemental table S3.1). One notable difference in the first round alone compared with the combined model is that the Thai *Ae. albopictus* did not present a significantly different probability of choosing human compared with the Cornell anthropophilic *Ae. aegypti* colony ($p=0.103$), although it remained significantly different from the Princeton anthropophilic *Ae. aegypti* colony ($p=0.007$). All other significant and non-significant

relationships remained the same as the combined model (α=0.05). In this round, the colonies exhibited a spectrum of response rates (percent of released mosquitoes that entered a host port), ranging from 11.2% to 25.0% for the *Ae. albopictus* colonies and between 41.6% and 71.3% for the *Ae. aegypti* colonies.

Results of the second round, which included the remaining four replicates, were also consistent with the combined model (Fig. 3.1C, Supplemental table S3.1). All nine *Ae. albopictus* colonies (the six original colonies and three biological replicates) were not significantly different from one another ($p>0.05$). The transport control (the Princeton anthropophilic *Ae. aegypti* colony reared at Cornell) did not behave differently from the same colony raised at Princeton ($p=1.00$), suggesting that preference behavior was not modified by the transport of mosquitoes from Cornell to Princeton and slight differences in rearing. The two Cameroonian colonies ($p=0.260$ and $p=0.2434$) and the zoophilic *Ae. aegypti* colony ($p=0.122$) were not different from the Cornell anthropophilic *Ae. aegypti*, but were different from the Princeton anthropophilic *Ae. aegypti*, as were all other *Ae. albopictus* colonies ($p<0.01$). All other significant and non-significant relationships remained the same as the combined model (α=0.05). In this round, the colony response rates ranged from 16.5% to 39.3% for the *Ae. albopictus* colonies and between 24.3% and 45.8% for the *Ae. aegypti* colonies

Variation by experimental subject

Individual host variation in mosquito attraction is a well-documented phenomenon[42]. To account for this, we examined mosquito behavioral responses across the individual experimental subjects (human and guinea pig). In the first round, a different human subject was used for each of the four replicates (Fig. 3.2). We detected significant differences in the predicted probability of choosing human between four of the six paired comparisons ($p<0.05$) and one pair with a

marginally significant difference (p=0.0514) (GLMM, human = fixed effect, colony = random effect).

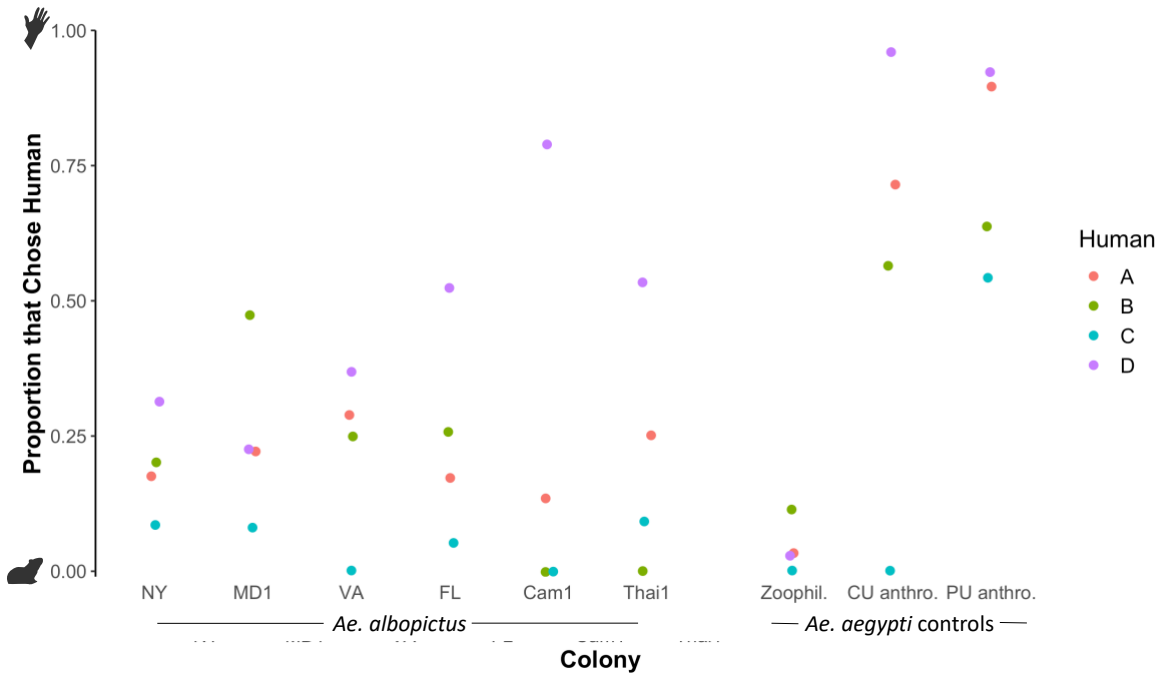


FIGURE 3.2: HOST CHOICE VARIATION BY HUMAN SUBJECT

This figure shows the proportion of host-seeking mosquitoes that chose human over guinea pig for each of the colonies. Each point represents the results of one replicate in the first round, with each color representing one of the four human subjects used in this round.

In the second round, the same human was used for all four replicates and two guinea pigs were used for two replicates each. This experimental set up allowed us to isolate the effect of guinea pig; one guinea pig was more attractive than the other ($p<0.0001$; Fig. 3.3) (GLMM, guinea pig = fixed effect, colony = fixed effect). When stratified by guinea pig, the pairwise estimated marginal mean comparisons between colonies for each guinea pig did not change the main conclusion that the *Ae. albopictus* colonies were less likely to choose human than

anthropophilic *Ae. aegypti* and behaved similarly to zoophilic *Ae. aegypti.* Although two guinea pigs were also used for two replicates each in the first round, each human was only paired with one guinea pig due to animal use constraints, making it difficult to isolate the effect of guinea pig, resulting in no significant difference between the two guinea pigs in the first round or combined models (p=0.065 and p=0.063, respectively).

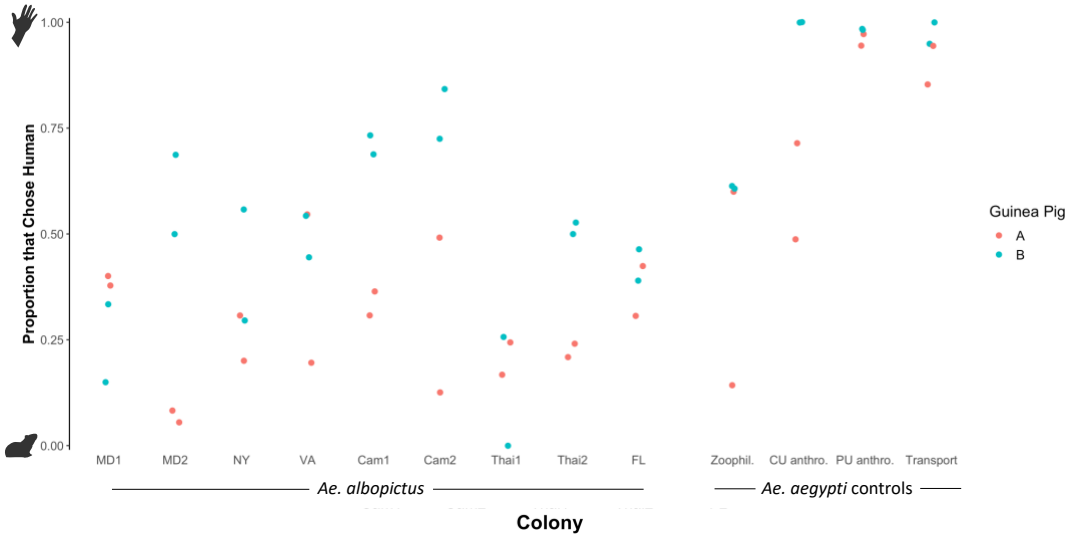


FIGURE 3.3: HOST CHOICE VARIATION BY GUINEA PIG SUBJECT

Scatter plot of the proportion of host-seeking mosquito that chose human over guinea pig for each of the colonies, with the color representing the guinea pig. Each point represents the results of one replicate in the second round.

Discussion

This study is the first to compare the host preference of *Ae. albopictus* across multiple populations, the first to characterize host preference of zoophagic *Ae. albopictus* populations, the first to assess *Ae. albopictus* host odor preference separately from other cues in a controlled

laboratory setting, and the first to directly compare host preference of *Ae. albopictus* to that of *Ae. aegypti.* Our results provide important insight into the behavior of this mosquito species and can help us to evaluate the relative importance of these vector species for transmission of anthroponotic and zoonotic pathogens.

Our results do not support the hypothesis that differences in *Ae. albopictus* host preferences drive observed differences in feeding patterns among populations in nature. The three anthropophagic *Ae. albopictus* populations we tested were not more likely to choose a human host *versus* a guinea pig compared with the three zoophagic populations. Further, we found no significant differences between any of the *Ae. albopictus* populations tested, representing a broad global distribution, including several populations from across the Eastern United States, Asia and Africa. Our results contrast with findings for two other species, including variation in *Ae. aegypti* host preference within Africa and between African and non-African populations, measured using the same experimental design we used in our study[11], and *Culex annulorostris* within Australia[43]. At a finer geographic scale, we also did not detect variation for host preference in the locations where we collected paired biological replicate colonies of *Ae. albopictus* within about 1.5km of one another, similar to the absence of fine scale variation between paired *Ae. aegypti* colonies collected within 5-10km of each other[11]. Given the wide distribution of *Ae. albopictus* around the world, it is possible that host preference variation exists at locations that were not included in this study. The colonies tested in our study do not encompass all genetic backgrounds identified globally[44].

All *Ae. albopictus* we tested, regardless of geographic origin and previously reported feeding patterns, were significantly less likely to choose human over guinea pig than the previously characterized Princeton anthropophilic *Ae. aegypti* from Thailand and none were

significantly different from the previously characterized zoophilic *Ae. aegypti* from Uganda[11]. Human preference responses between our two anthropophilic *Ae. aegypti* colonies varied but were not significantly different.

Our results suggest that *Ae. albopictus* host preference behavior is closer to that of ancestral *Ae. aegypti* populations than to that of the invasive *Ae. aegypti* lineage that evolved to specialize in biting humans and then spread out of Africa and around the world. We can therefore conclude that *Ae. albopictus* is less anthropophilic than invasive *Ae. aegypti.* In the past, *Ae. albopictus* has been considered both an anthropophilic and a generalist blood feeder[1]; however *Ae. albopictus* has typically been presumed to be less anthropophilic than *Ae. aegypti* (e.g.[45]). The quantification of their relative anthropophily in this study has implications for understanding the relative threat posed by the two species, which transmit many of the same pathogens (e.g. dengue, Zika, and chikungunya) and live in similar habitats with overlapping distributions[46]. *Aedes albopictus* may only pose a comparable threat for transmission of anthroponoses in settings where humans are highly available, such as densely populated urban areas with open housing structures. It also suggests that in settings where humans are available at intermediate levels alongside other hosts, *Ae. albopictus* is more likely to transmit zoonotic pathogens than *Ae. aegypti*.

Our assays also demonstrated variation in preference for host individuals of the same species (both individual humans and individual guinea pigs). It is well-established that there is variation in mosquito attraction to different humans (reviewed by Martinez et al., 2021[42]). It is notable that variation in human preference was observed in the first round of our experiment despite the recruitment process to select humans with a relatively high level of attractiveness to anthropophilic *Ae. aegypti*, which may have been expected to limit the observed variation in

attraction among human hosts. We do not know whether *Ae. aegypti* and *Ae. albopictus* prefer the same individual hosts. It is possible that *Ae. albopictus* responds to different odor components than *Ae. aegypti,* which would mean that human subjects chosen to maximize the response of anthropophilic *Ae. aegypti* (see Methods) might create or increase the perceived difference in human preference between the two species in the first experimental round. Mosquito species can respond differently to bacterial volatiles from different host species[47], which may also be the case for differences between individuals of a given host species.

Variation in intra-species attraction among non-human hosts has been reported previously, with attraction varying by physiological stage in rats[48], stress hormones in zebra finches[49], and body mass and metabolic rate in house sparrows[50]. The two guinea pig hosts used in our study were both mature females, however they were different ages, with the older of the two being the more attractive. We did not measure body mass, metabolic rate, stress hormones, or any other parameter that may have driven the difference in attraction of the two guinea pigs. Additional experiments designed to assess attraction to individual hosts may provide more insight on variation in attractiveness among individual humans and among individual non-human hosts. Many host preference studies use one or a few individuals to represent the human and non-human host species; it should be acknowledged that the individuals chosen for each species may impact the magnitude and even directionality of the measured preference.

In our study, the way human odor was presented to the mosquitoes differed between round one and two. In round one, air was passed over only the middle section of a human arm, from just below to just above the elbow (Fig. 3.4). In round two, air was passed over the full forearm and hand. We found that changing the arm display from the elbow to the full forearm and hand could increase the level of attractiveness of an unattractive human subject. Exposing

the full forearm and hand may have increased available surface area or exposed different odors emitted by different parts of the arm. Some mosquito species exhibit preference for certain body parts, based on preference for specific microbial volatiles in those areas[51], although such body part-specific preferences are not always detected[52]. Despite this change in odor presentation, the patterns of host attraction remained similar across the first and second rounds.

In addition to the challenge of individual host variation in attractiveness, laboratory preference assays need to ensure field-relevance of the mosquito colonies. Collection and colony rearing techniques can lead to founder effects, bottlenecks, and selection[53,54]. We attempted to limit selection pressure on host preference by sampling *Ae. albopictus* population egg or larval stages, except for the Thai *Ae. albopictus,* which were collected via human-landing catch, potentially selecting for human preference. Despite this difference, the Thai *Ae. albopictus* colonies were not more likely to choose human than the other colonies when tested in the olfactometer. To avoid laboratory selection for host preference, as has been described in other species[55], mosquitoes were tested within just a few generations of colony establishment (F2-F4), were fed on artificial feeders with minimal host cues, and were given ample time to blood feed (until >90% were fed). Therefore, we expect that the laboratory colonies tested in these experiments are representative of the field populations.

The goal of our study was to understand, for the first time, levels of *Ae. albopictus* anthropophily across global geographic isolates. Our experimental design exposed a small but consistent part of human hosts to mosquitoes and was conducted in a controlled laboratory setting. We chose to use guinea pig as the non-human host because it is one of the most common hosts used in *Ae. aegypti* host odor preference assays, allowing us to assess *Ae. albopictus* host preference using a host that elicits replicable, divergent behavior between the *Ae. aegypti* control

colonies. Domesticated guinea pigs have been dispersed by trade throughout much of the world from their native origin in South America[56], resulting in an overlapping geographic distribution with the globally invasive *Ae. albopictus.* Although guinea pig has never been reported in *Ae. albopictus* blood meal contents, it is unknown whether guinea pigs were present at the study sites. As such, it is possible that guinea pigs serve as a natural host in the field, but it has yet to be demonstrated. Further research is needed to understand the probability of choosing a human over more relevant non-human animals in field settings given large variation in host sizes and the need to include a range of hosts naturally available to *Ae. albopictus*. Our results are in contrast to the two previous assessments of *Ae. albopictus* host preference, which tested the full human body versus various non-human animals and concluded that *Ae. albopictus* prefer humans to non-human animals[14,15]. In the latter study, host defenses may have contributed to the conclusion of human preference. Additional *Ae. albopictus* host preference assays should be conducted using different experimental techniques and host animals to better understand this trait and tease out potential artifacts of experimental design.

Here, we demonstrated for the first time that *Ae. albopictus* host preference is not likely to be the driver of the highly variable feeding patterns reported in the literature for this species. The *Ae. albopictus* colonies that we tested were consistently zoophilic in contrast to the highly anthropophilic invasive lineage of *Ae. aegypti,* further supporting our understanding that *Ae. aegypti* has a higher capacity to transmit arboviruses between humans than *Ae. albopictus*.

Methods

Colony Establishment

Based on previously reported *Ae. albopictus* blood meal analysis studies, three populations with high levels of anthropophagy and three populations with low levels of anthropophagy were selected. The high anthropophagy populations included Cameroon (99.4% anthropophagy, defined as the percent of all identified bloodmeals identified as human)[41], Thailand (100% anthropophagy, including 5.7% that fed on both a human and non-human animal)[35], and Florida, USA (91% anthropophagy)[40]. The low anthropophagy populations, all from the eastern United States, included New York (32.2%)[17], Maryland (13.6%)[57], and Virginia (7.3%)[39]. Three of these populations (Cameroon, Thailand, and Maryland) included a biological replicate (a second colony from a site at least 1.5km away from the primary colony collection site). The collections took place at roughly the same sites as the blood meals were collected in previous studies, except for Thailand because sites could not be reached due to COVID travel restrictions (see Supplemental document 1 for site details). However, time gaps did exist between the blood meal and colony collections, ranging from 18 to 2 years (Thailand and Virginia, respectively). Populations may have evolved in the intervening time, potentially reducing the concordance between population traits that produced the feeding patterns and the host preferences observed here.

Most collections were conducted with oviposition traps, black buckets treated with an attractant (water infused with dog food, hay, or other organic material, depending on site) and lined with paper towel or seed germination paper to collect eggs. At least ten traps were distributed at least 100m apart, except for the Florida site, which was in a scrapyard that was not sufficiently large for such distant spacing. Collections were conducted over 2-3 weeks and egg sheets were removed multiple times per week. The collections that were not conducted with oviposition traps included Thailand, Cameroon, and one of the Maryland biological replicates

(Maryland 2). In Thailand, collections were conducted with human landing catches, which were performed prior to other collections and could not be repeated with the standardized collection methods (designed to avoid selection on host preference) due to COVID travel restrictions. In Cameroon and the Maryland biological replicate, larval collections were conducted. Larvae were collected from at least 10 containers at least 100 m apart.

The control colonies were established prior to this study. The zoophilic *Ae. aegypti* was originally collected in Uganda (ZIK in Rose et al. 2020)[11], and both the Princeton (T51 in Rose et al. 2020)[11] and Cornell[58] anthropophilic *Ae. aegypti* were originally collected in Thailand.

Mosquito Rearing

In the case of oviposition collections, egg sheets were sent directly to Cornell University, where they were maintained in an environmental chamber until hatching (28°C, 71.9% ± 9.5% relative humidity, 10 hour light, 10 hour dark, and 2 hour dusk/dawn). Egg sheets were soaked in water for 20 minutes and then vacuum-hatched and provided with a pinch of pulverized fish food (medium Cichlid Gold™ fish food pellets; Hikari, Himeji, Japan). Within 24 hours, larvae were transferred from the hatch flask to rearing trays, with 200 larvae, 1 liter of distilled water, and 7 fish food pellets in each. Upon pupation, mosquitoes were transferred to cups and placed in cages. Adult females were blood fed using an artificial feeding system, with human blood (Valley Biomedical, Winchester, VA, USA), with sausage casing as the piercing membrane (DCW Casing LLC, Mount Vernon, NY, USA). Colonies were fed at approximately 7, 14, and 21 days post-eclosion. To avoid selection during the feeding process, colonies were monitored to ensure high rates of feeding (>90%) and were fed a second day if sufficient feeding levels were not reached. Three days post-feeding, oviposition cups were placed into the cage, treated with

strained larval water to induce improved egg-laying[59] and dirt in the case of the Ugandan colony. Three days later, oviposition cups were removed and egg sheets were dried until slightly damp and then maintained in the environmental chamber. All colonies derived from oviposition traps were maintained in colony in this form until they were used in the preference assays in generation F2, except for the primary Maryland colony, which was F3 in the second experimental round.

In Thailand, the adult female mosquitoes captured through human landing catch were brought to the lab and blood fed using human blood via an artificial feeder. The egg sheets derived from these feedings were sent to Cornell University (F1). The Thai *Ae. albopictus* used in experiments were generation F3. In Cameroon, the collected larvae were brought to the lab, reared, and blood fed using rabbit blood (live and via artificial feeder). They were maintained in colony in Cameroon for one more generation and F2 eggs were sent to Cornell University. The Cameroonian *Ae. albopictus* used in experiments were generation F3 and F4. For the second biological replicate from Maryland, larvae were shipped in water to Cornell University. The *Ae. albopictus* from the Maryland biological replicate colony was generation F2 in the experiments. In all cases, upon arrival at Cornell University, the colonies were maintained as described above until preference assays were performed.

The zoophilic *Ae. aegypti* eggs were sent to Cornell from a colony at Princeton and reared as described above. The Cornell anthropophilic *Ae. aegypti* were acquired directly from eggs derived from the Cornell colony, which is maintained similarly to the methods described above, except for blood feeding, which is typically conducted with a live human and periodically with a live chicken. Mosquitoes reared for experiments were reared in the same fashion as the *Ae. albopictus* colonies. The Princeton anthropophilic *Ae. aegypti* were reared at Princeton (F14),

with slight differences in rearing protocol: eggs were hatched in deoxygenated water and fed Tetramin Tropical Tablets fish food (Spectrum Brands, Inc) *ad libitum* until pupation, then transferred to cages. In the second experimental round, the Princeton colony was reared at Cornell as well as Princeton as a transport control - eggs were brought to Cornell and reared alongside the other colonies.

At about five days post-eclosion, colonies were transported to Princeton in a heated car for preference assays (except for the Princeton *Ae. aegypti*, which were already located there). No mortality was noticed. The colonies were given approximately 50 hours to acclimate in the rearing chamber at 28°C (71.9% ± 9.5% RH) before preference assays commenced.

Preference Assays

First-round

Preference assays were performed in a dual-port olfactometer (Fig. 3.4a), using a similar methodology as previous studies[11,13]. Between 50-175 females were released in the mosquito chamber and allowed to acclimate for approximately ten minutes. Only the first and second replicate of the two anthropophilic *Ae. aegypti* colonies were conducted with fewer than 98 females; all others included 98 -175 females. After acclimation, the ports were opened and the wind source was turned on, moving carbon-filtered air over the hosts and into the mosquito chamber, presenting the mosquitoes with a human and guinea pig odor plume emanating from the respective ports. The human odor source included the elbow of the human, inserted perpendicularly through two holes in the host chamber, and breath, exhaled from the nose every 30 seconds through a breathing tube (Fig. 3.4b). The full guinea pig was presented in the other host chamber and allowed to breathe normally. Guinea pig was chosen as the non-human host

because it is one of the most common hosts used to assess *Ae. aegypti* host odor preference, eliciting replicable, divergent behavior between the control colonies[11]. The mosquitoes were given ten minutes to fly upwind and enter one of the port traps, where they were prevented from accessing the host via a screen and inhibited from flying back into the mosquito chamber via a funnel. At the conclusion of the ten minutes, the number of mosquitoes that entered each port was counted.

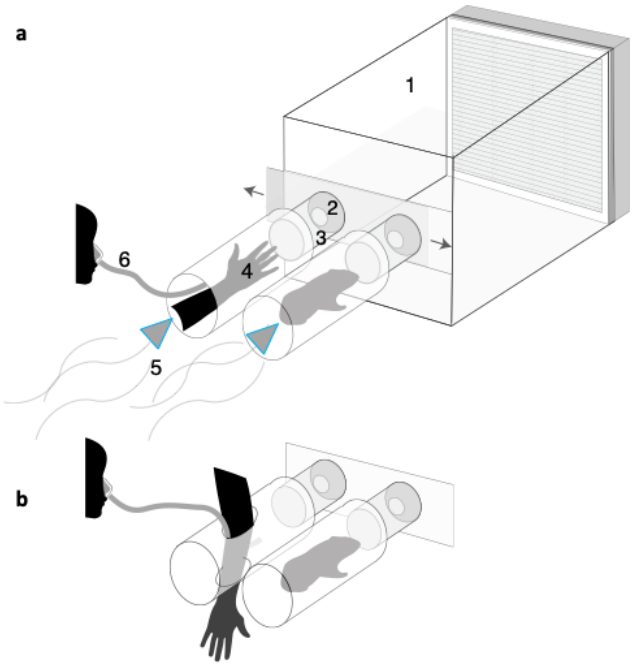


FIGURE 3.4: DUAL-PORT OLFACTOMETER AND ARM PRESENTATION

a. The dual-port olfactometer consists of the following components: 1. the mosquito chamber, in which mosquitoes are released and allowed 10 minutes to acclimate prior to odor exposure; 2. funnel that allows mosquito entry into ports after the sliding door is opened but limits return of mosquitoes from the trap to the mosquito chamber; 3. mosquito trap, with a funnel on one end and a screen on the other to prevent access to hosts; 4. host chambers that hold the host for odor presentation; the method of human arm presentation pictured here was used in the second experimental round (the full forearm and hand were inserted through the far end of the tube and exposed to air flow, with the breathing tube inserted alongside the arm). An opaque panel (not pictured) divided the hosts from the mosquitoes such that they were not visible from the mosquito

chamber prior to host choice; 5. wind source, which was created by blowing a carbon-filtered air source over the hosts in the first round (with a nozzle connected to each host chamber and an air outtake at the other end of the mosquito chamber; ~0.6m/s windspeed), and in the second round, created by a window fan fitted to the far end of the mosquito chamber, producing air flow by sucking air from the room through the host chambers and the mosquito chamber. **b.** In the first experimental round, the human arm was displayed by inserting the arm perpendicularly through the host chamber, with the hand protruding from the opposite side, such that the elbow was exposed to air flow. The breathing tube (6) was also inserted into the host chamber. Schematics modified from Metz et al. (2022)[64].

The first round of replicates was conducted over four days. Each day, the six primary *Ae. albopictus* colonies, the zoophilic *Ae. aegypti*, and the two anthropophilic *Ae. aegypti* colonies were tested. The order of testing was rotated so that the colonies would be tested at different times of day. The side on which the human and guinea pig were presented was swapped after approximately half of the replicates were completed each day.

Potential human subjects were tested for attractiveness level prior to inclusion using the two anthropophilic *Ae. aegypti* colonies; the goal of this study was to measure the relative anthropophily of the colonies, so we wanted to include attractive humans to maximize the potential dynamic range of anthropophily observed. We selected four of seven tested human subjects; two were excluded due to low levels of attraction and one due to illness during the first round. Each of the four human subjects were used for one full day of replicates (all nine colonies tested in this round). These participation of humans in olfactometer trials using these methods was approved and monitored by the Princeton University Institutional Review Board (protocol 8170). All participants provided informed consent before participating.

Two guinea pigs were used and rotated after each day of experiments. The guinea pigs periodically defecated or urinated during the trial; when this occurred, the soiled protective sheet at the bottom of both host chambers were removed and replaced with new sheets. The use of guinea pigs in olfactometer trials was approved and monitored by the Princeton University Institutional Animal Care and Use Committee (protocol 1998-20). All methods were carried out in accordance with the corresponding guidelines and regulations.

Second Round

The second experimental round was performed largely the same as the first round with several exceptions. In the second round, personnel constraints required one human to serve as the human subject for all four replicates, with the added benefit of removing one potential source of variation. However, the human subject in question was deemed relatively unattractive based on the recruitment assays that were conducted prior to the first round and was excluded from that round. Based on anecdotal experience, a pilot was conducted comparing two forms of arm display: the elbow as in the first round (Fig. 3.4b) versus the full forearm and hand (Fig. 3.4a). This demonstrated an increase in the probability of choosing human using the full forearm and hand compared with the elbow, which was later confirmed by a small trial (Supplemental document 2). Changing the arm presentation also required changing the airflow system. The arm was inserted through the back of the human host chamber, preventing the connection of the carbon-filtered air system. Instead, the fan attached to the far end of the mosquito chamber was alone responsible for drawing air from the room over the hosts and through the mosquito chamber, as done in a previous study[13]. This may have caused more mixing of host odors; however, efforts were made to reduce this phenomenon: the air exchange in the room holding the

dual-port olfactometer is extremely high (multiple exchanges per hour) and the hosts were removed from the room for at least 15 minutes between each replicate, limiting the accumulation of host odors in the room.

The other difference in the second round was that the three biological replicate colonies and a transport control were included in addition to the nine colonies tested in the previous round. As a result, thirteen colonies were tested each day. The transport control was added to assess whether the transport from Cornell to Princeton impacted the host seeking behavior. The Princeton anthropophilic *Ae. aegypti* were reared at both Princeton and Cornell.

Data Analysis

A Generalized Linear Mixed Model using Template Model Builder (glmmTMB)[60] with a beta-binomial distribution was employed to evaluate the contribution of several factors to the predicted probability of choosing human for each of the analyses described below (the combined, first round, and second round models that seek to evaluate the effect of colony and the human model, which seeks to evaluate the effect of human). Post hoc analyses were conducted by calculating the estimated marginal means of the effects by using emmeans[61]. A predicted probability of choosing human equivalent to 0.5 indicates no preference between human and guinea pig; between 0.5 and 1 indicates a preference for human and between 0 and 0.5 indicates a preference for guinea pig. Analyses were conducted using R version 4.1.1[62]. Graphs were created using ggplot[63].

Combined model

The data for the six primary *Ae. albopictus* colonies, the zoophilic *Ae. aegypti* and the two anthropophilic *Ae. aegypti* colonies were combined across eight replicates conducted in the first and second experimental rounds, which we refer to as the combined model. In this model, colony, guinea pig, and side of human host chamber were included as fixed effects and with random effects of human and date.

First-round model

In this model, colony, guinea pig, and side of human host chamber were included as fixed effects and human as a random effect. Each human was only tested one day in the first round, so date was excluded from this analysis.

Second-round model

In this model, colony, guinea pig, and side of human host chamber were included as fixed effects and date as a random effect. Only one human was tested in the second round, so human was excluded from this analysis.

Human model

To evaluate the differential attraction to each of the four humans in the first round, human and side of human host chamber were included as fixed effects and colony as a random effect. We did not include guinea pig in this analysis, because each human was only tested against one guinea pig, resulting in nesting of this data.

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Data Availability: Data will be deposited in Cornell University Library's institutional repository, eCommons (https://ecommons.cornell.edu) for preservation and access via the world wide web without restriction at the following DOI: https://doi.org/10.7298/k8wf-bh80.

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SUPPLEMENTAL TABLE S3.1: PREDICTED PROBABILITY OF CHOOSING HUMAN FOR EACH *AE. ALBOPICTUS* AND *AE. AEGYPTI* COLONY BASED ON GENERALIZED LINEAR MIXED MODELS WITH DATA COMBINED AND FOR EACH EXPERIMENTAL ROUND SEPARATELY

Colony	Combined			First Round			Second Round		
	Probability	SE	Sig*	Probability	SE	Sig*	Probability	SE	Sig*
Ae. albopictus									
New York	0.258	0.071	A	0.193	0.0803	A	0.346	0.0654	A
Maryland 1	0.254	0.071	A	0.199	0.0779	A	0.314	0.0637	A
Maryland 2	—‡	—	—	—	—	—	0.293	0.0693	A
Virginia	0.304	0.0779	A	0.2051	0.0822	A	0.425	0.0691	A
Florida	0.306	0.0764	A	0.1961	0.0765	A	0.404	0.0709	A
Cameroon 1	0.386	0.0866	A	0.2463	0.0976	A	0.557	0.0679	AB
Cameroon 2	—	—	—	—	—	—	0.561	0.0699	AB
Thailand 1	0.202	0.0672	A	0.2612	0.1075	AB	0.199	0.0597	A
Thailand 2	—	—	—	—	—	—	0.338	0.0737	A
Ae. aegypti ξ									
Zoophilic	0.206	0.0614	A	0.0385	0.0239	A	0.506	0.0751	AB
Cornell anthropophilic	0.735	0.0711	B	0.6408	0.2816	BC	0.818	0.0527	BC
Princeton anthropophilic	0.877	0.0432	B	0.7959	0.0785	C	0.957	0.0211	C
Transport Control	—	—	—	—	—	—	0.947	0.0248	C

*groups with different letters within a column represent colonies with significantly different probabilities of choosing human according to GLMM with Tukey post-hoc test, using glmmTMB and emmeans R packages.

‡colonies with dashes in the Combined and First Round columns were not assessed in the first experimental round and are therefore also not included in the combined model.

ξzoophilic is a Ugandan colony, Cornell anthropophilic is a Thai colony established and reared at Cornell, Princeton anthropophilic is a Thai colony established and reared at Princeton, and Transport Control is the Princeton anthropophilic colony reared at Cornell.

Supplemental Document 1

Below are the details for the origin of each *Aedes albopictus* colony.

1) **New York**
    a. **Location:** Babylon, New York, USA (40.69, -73.33)
    b. **Collection method:** 22 ovitraps, 2 placed at each of 11 properties.
    c. **Dates of collection:** July 19 – 31, 2021
2) **Virginia**
    a. **Location:** Suffolk, Virginia, USA (36.73, -76.58)
    b. **Collection method:** 10 ovitraps, 1 placed at each of 10 properties
    c. **Dates of collection:** July 19 – July 29, 2021
3) **Maryland 1**
    a. **Location:** Harlem Park (39.29, -76.63) and Hollins Market/Union Square (39.28, -76.62) neighborhoods, Baltimore, Maryland, USA
    b. **Collection method:** Ovitraps; 1 ovitrap placed at each of 13 properties in Harlem Park and 1 ovitrap at each of 15 properties in Hollins Market/Union Square neighborhood. These neighborhoods were originally meant to be biological replicates, but due to low hatch rate, were combined into one colony
    c. **Dates of collection:** July 6 – 13, 2021
4) **Maryland 2**
    a. **Location:** Bolton Hill neighborhood, Baltimore, Maryland, USA (39.30, -76.62)
    b. **Collection method:** Larval collections; approximately 100 larvae were collected from around 20 containers spread over 5-6 city blocks.
    c. **Dates of collection:** August 31, 2021

5) **Florida**
    a. **Location:** Scrap yard, Vero Beach, Florida, USA (27.67, -80.43)
    b. **Collection method:** 10 ovitraps spread out within the scrapyard property
    c. **Dates of collection:** June 28 – July 26, 2021
6) **Cameroon 1**
    a. **Location:** Suburban neighborhood in Yaoundé, Cameroon
    b. **Collection method:** Larval collections from approximately 30 containers
7) **Cameroon 2**
    a. **Location:** Downtown Yaoundé, Cameroon
    b. **Collection method:** Larval collections from approximately 30 containers
8) **Thailand 1**
    a. **Location:** Ban Bueng District, Chon Buri, Thailand (13.263841, 101.136787)
    b. **Collection method:** 250 females were collected via human landing catch from 10-15 spots in vegetated areas, rubber tree plantations, and fruit orchards, with each spot about 20m or more apart.
9) **Thailand 2**
    a. **Location:** Pluak Daeng District, Rayong, Thailand (12.946102, 101.311641).
    b. **Collection method:** 151 females were collected via human landing catch from 10-15 spots in vegetated areas, rubber tree plantations, and fruit orchards, with each spot about 20m or more apart.

Supplemental document 2

We conducted a pilot comparison between two methods of human arm presentation, the elbow versus the forearm, to test whether a different method of presentation would increase attraction to a human who is deemed unattractive using the elbow presentation. This pilot was promising, showing markedly increased attraction of the Princeton anthropophilic *Ae. aegypti* to a human who attracted very few mosquitoes during the subject selection trials. We conducted a small trial to confirm this observation, testing both the Princeton anthropophilic *Ae. aegypti* and zoophilic *Ae. aegypti* colonies with both the forearm and elbow arm presentation methods. Three replicates were conducted for each replicate/presentation combination (Figure 3.5a). The results were analyzed with a generalized linear mixed model using a betabinomial distribution, with group and human side as fixed effects. We confirmed that the forearm method of presentation indeed led to an increase in attraction of the anthropophilic *Ae. aegypti* to a relatively unattractive human compared with the elbow presentation ($p = 0.0197$, Figure 3.5b). The zoophilic *Ae. aegypti* colony was also more attracted to the human with the forearm presentation method compared with elbow, but not significantly ($p = 0.0650$). Notably, the anthropophilic and zoophilic colonies did not have a significantly different probability of choosing human within each arm presentation type (forearm $p=0.2175$; elbow $p=0.9777$). This is in contrast to the significant differences that were found between these groups in both round one, round two, and combined model analyses. With only three replicates per group in this small experiment, there was not sufficient sample size to detect colony differences. However, that was not the goal of this trial, which was to show the difference in arm presentation method.

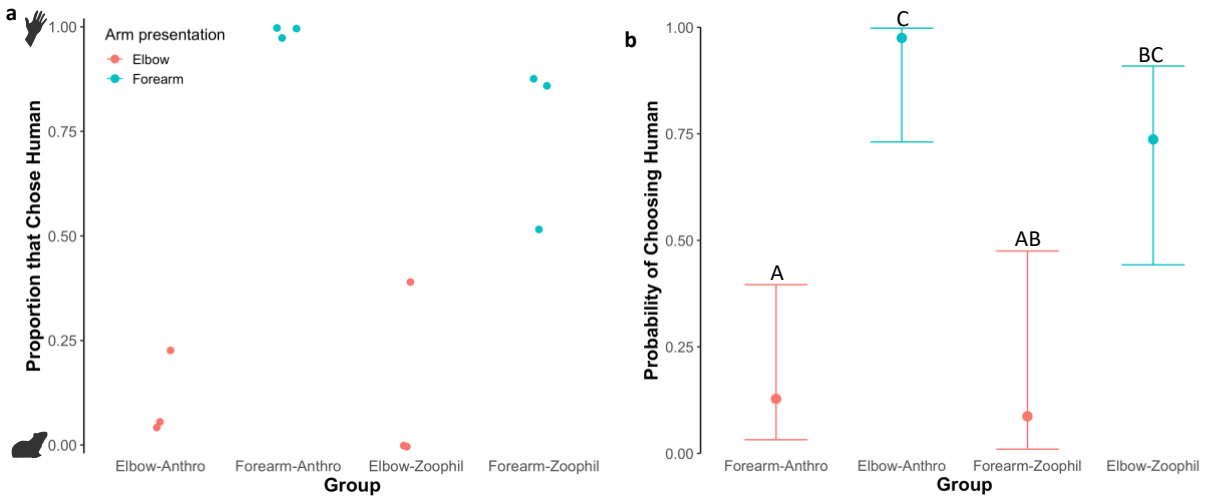

FIGURE 3.5: EFFECT OF ARM PRESENTATION ON HOST CHOICE

a) This scatter plot shows the proportion of mosquitoes that chose human over guinea pig for each group. Each point represents the results of one replicate, with color representing arm presentation method. Each colony and arm presentation combination is represented on the x axis; the "Anthro" colonies are the Princeton anthropophilic *Ae. aegypti* and the "Zoophil" colonies are the zoophilic *Ae. aegypti*. **b)** This graph shows the predicted probability of choosing human for each group (Tukey post-hoc analysis of GLMM, using glmmTMB and emmeans packages), with the bars showing the upper and lower confidence limits. The letters over each group represent statistical significance, with groups that do not share a letter having significantly different probabilities of choosing human ($p<0.05$)

Chapter 4 : Sugar feeding patterns of New York *Aedes albopictus* mosquitoes are affected by saturation deficit, flowers, and host seeking[*]

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Abstract

Background

Sugar feeding is an important behavior which may determine vector potential of female mosquitoes. Sugar meals can reduce blood feeding frequency, enhance survival, and decrease fecundity, as well as provide energetic reserves to fuel energy intensive behaviors such as mating and host seeking. Sugar feeding behavior can be harnessed for vector control (e.g. attractive toxic sugar baits). Few studies have addressed sugar feeding of *Aedes albopictus,* a vector of arboviruses of public health importance, including dengue and Zika viruses. To address this knowledge gap, we assessed sugar feeding patterns of *Ae. albopictus* for the first time in its invasive northeastern USA range.

Methodology/ Principal Findings

[*]Presented with minor modifications from the originally published article: Fikrig, K. et al. Sugar feeding patterns of New York *Aedes albopictus* mosquitoes are affected by saturation deficit, flowers, and host seeking. *PLoS Neglected Tropical Diseases* 14, 16, doi:10.1371/journal.pntd.0008244 (2020).

Using the cold anthrone fructose assay with robust sample sizes, we demonstrated that a large percentage of both male (49.6%) and female (41.8%) *Ae. albopictus* fed on plant or homopteran derived sugar sources within 24 hrs prior to capture. Our results suggest that sugar feeding behavior increases when environmental conditions are dry (high saturation deficit) and may vary by behavioral status (host seeking vs. resting). Furthermore, mosquitoes collected on properties with flowers (>3 blooms) had higher fructose concentrations compared with those collected from properties with few to no flowers (0-3).

Conclusions/Significance

Our results provide the first evidence of *Ae. albopictus* sugar feeding behavior in the Northeastern US and reveal relatively high rates of sugar feeding. These results suggest the potential success for regional deployment of toxic sugar baits. In addition, we demonstrate the impact of several environmental and mosquito parameters (saturation deficit, presence of flowers, host seeking status, and sex) on sugar feeding. Placing sugar feeding behavior in the context of these environmental and mosquito parameters provides further insight into spatiotemporal dynamics of feeding behavior for *Ae. albopictus*, and in turn, provides information for evidence-based control decisions.

Author summary

Sugar feeding on plant nectar and other sources is an important mosquito behavior that varies between mosquito types. It is critical to understand sugar feeding because it impacts other aspects of mosquito biology, such as egg production, survival, and energy for activities such as mating and host seeking. Sugar can also be used to trap and kill mosquitoes. For example,

attractive toxic sugar baits have been tested as a new control technique that depends on sugar feeding behavior for success. We investigated this behavior for the Asian tiger mosquito, a globally invasive species that can transmit several pathogens. We know very little about its sugar feeding behavior – only 4 studies have been conducted on the topic prior to ours, and none in Northeastern US, where our study was conducted. We found that hot and dry weather leads the mosquito to sugar feed more often and the presence of flowers increases the amount of sugar contained in those mosquitoes. Unexpectedly, we observed that host-seeking mosquitoes were more likely to be sugar fed than resting mosquitoes, which is contrary to previous studies showing a reduction in blood feeding after sugar feeding. In order to fully understand the patterns that we observed, further research will be necessary.

Introduction

Aedes albopictus is a vector of numerous pathogens, including dengue, chikungunya and Zika viruses as well as dog heartworm parasites [1-3]. Its global range is rapidly expanding and pushing northward in the USA, enabled by local adaptation and winter egg diapause [4, 5]. This highly adaptable mosquito can survive in drastically varied ecosystems, ranging from tropical to temperate climates, making it one of the most successful invasive species globally [6]. Understanding this mosquito's feeding behavior and ecology across its invasive range is essential for understanding risk and devising control methods.

Sugar feeding is an important mosquito behavior with implications for disease transmission and control [7]. It can impact mosquito life history through a number of mechanisms and can vary between mosquito species [8]. For females, there may be trade-offs in transmission potential between blood and sugar feeding [8], as the latter may lead to satiation

and reduce available abdominal space for a blood meal shortly after sugar feeding [9]. Sugar feeding has been considered a blood feeding suppressant in *Ae. albopictus* and other mosquito species, reducing blood meal size and frequency, thereby reducing opportunities for pathogen transmission [8, 10, 11]. In contrast, sugar feeding can enhance survival of *Ae. albopictus* and other mosquito species in laboratory studies, potentially increasing pathogen transmission [8, 10, 12-17]. Sugar also may enhance male mosquito mating performance by providing energy for mate-seeking and swarming [13, 17-20] and enable female host-seeking behavior [20]. In addition to impacts on mosquito life history, sugar feeding behavior has implications for the success of certain control and surveillance methods, such as attractive toxic (or targeted) sugar baits (ATSBs), which contain sugar and flower-derived attractants mixed with insecticides [21].

Environmental drivers that vary widely across *Ae. albopictus* invasive range can influence feeding behavior. For example, fructose feeding rates can vary by season and location, which may be caused by differences in temperature and humidity [22-26]. Dehydration due to low humidity conditions may stimulate sugar feeding behavior as has been shown by Hagan et al. (2018) for blood feeding [27]. Availability of sugar sources such as floral nectar may also affect mosquito sugar feeding rates, especially in arid climates [24, 28-30]. However, in addition to flower nectar, mosquitoes can acquire sugar from plant leaves, fruit, and homopteran honeydew [15, 24, 25, 31, 32] and these alternative sources can vary across *Ae. albopictus* habitats.

Given the importance of sugar feeding for mosquito fitness and the public health threat of *Ae. albopictus,* we know surprisingly little about its sugar feeding patterns in nature. Only four field studies have been conducted across vastly different habitats [17, 24, 33, 34]. Two studies

indicated that season, habitat, and sugar availability might be important, as well as temperature and humidity [17, 24].

In Israel, the percent of sugar positive *Ae. albopictus* varied by season and habitat type (irrigated garden versus dry wasteland), ranging from 41.4% (summer) to 74.1% (fall) at a wasteland site [24]. However, this study was performed with an abbreviated cold anthrone assay, using visual detection of color change, instead of precise measurement of fructose concentration using established methods [24]. A subsequent study evaluating *Ae. albopictus* visitation to sugar sources reported attraction to a subset of tested ornamental flowers, wildflowers, damaged carob seed pods and fruits, but no attraction was detected to honeydew coated plants [34]. Working with releases of laboratory colony males (F_{33}-F_{47}) in northern Italy, Bellini et al. (2014) utilized an abbreviated cold anthrone assay to detect higher sugar feeding rates for released males at sites with sucrose feeding stations compared with control sites and a positive correlation with temperature and negative correlation with humidity [17]. In Florida, where the only other US study was conducted, *Ae. albopictus* fructose concentration did not vary significantly with plant species utilized as resting habitat; unfortunately, no analyses were conducted to determine the proportion sugar fed [33]. Another limitation of these studies was the lack of established baseline fructose levels, leading to the potential misidentification of larval nutrients as adult sugar meals.

The current knowledge of *Ae. albopictus* sugar feeding in the field primarily stems from these four studies in Israel, Italy, and Florida. Additionally, assessments of ATSBs for *Ae. albopictus* population control in Florida and Israel have demonstrated that sugar feeding frequency is sufficient to achieve population reductions [35-39]. However, these locations are not representative of the vast environmental variation in climate and flora where *Ae. albopictus* is now established. It has yet to be determined whether sugar feeding behavior of *Ae. albopictus*

in other regions of the world will be conducive to ATSB success due to an absence of basic ecological and behavioral information on this subject.

To address this important gap, we assessed the sugar feeding behavior of *Ae. albopictus* at its invasive edge in Northeastern USA in order to understand its feeding ecology along the northern limit of its expanding range. We determined the proportion of male and female mosquitoes that contained fructose and individual mosquito fructose concentrations. In addition, we assessed the response of sugar feeding to saturation deficit (environmental dryness), floral presence, host seeking status, and sex. Placing sugar feeding behavior in the context of these environmental and mosquito parameters provides further insight into spatiotemporal dynamics of this behavior for *Ae. albopictus*, and in turn, provides information for evidence-based control decisions.

Methods

Field Site:

Mosquitoes were collected in Long Island, New York, USA, at four farms and four residential areas with 9-17 houses in each, totaling 50 properties. Sites were chosen based on prior knowledge of *Ae. albopictus* distribution in Suffolk County from larval surveys and vector control surveillance [40](S. Campbell, pers comm). The eight sites were located in separate towns spanning a substantial section of Long Island (40km East to West and 15km North to South) (Figure 4.1). All four farms were surrounded to some degree by both forested and residential land. The four residential areas had variable levels of vegetation, both within and between sites. Residential property sizes ranged from approximately 200 – 1200m^2. Terrain was flat across all 8 sites (elevation range approximately 3 - 79m above sea level). Collections were

conducted between June and August 2018. Two HOBO Pro v2 data loggers (model U23-001, Onset Computer Corp., Bourne MA, USA) per site recorded the temperature and humidity every four hours from mid-July through August.

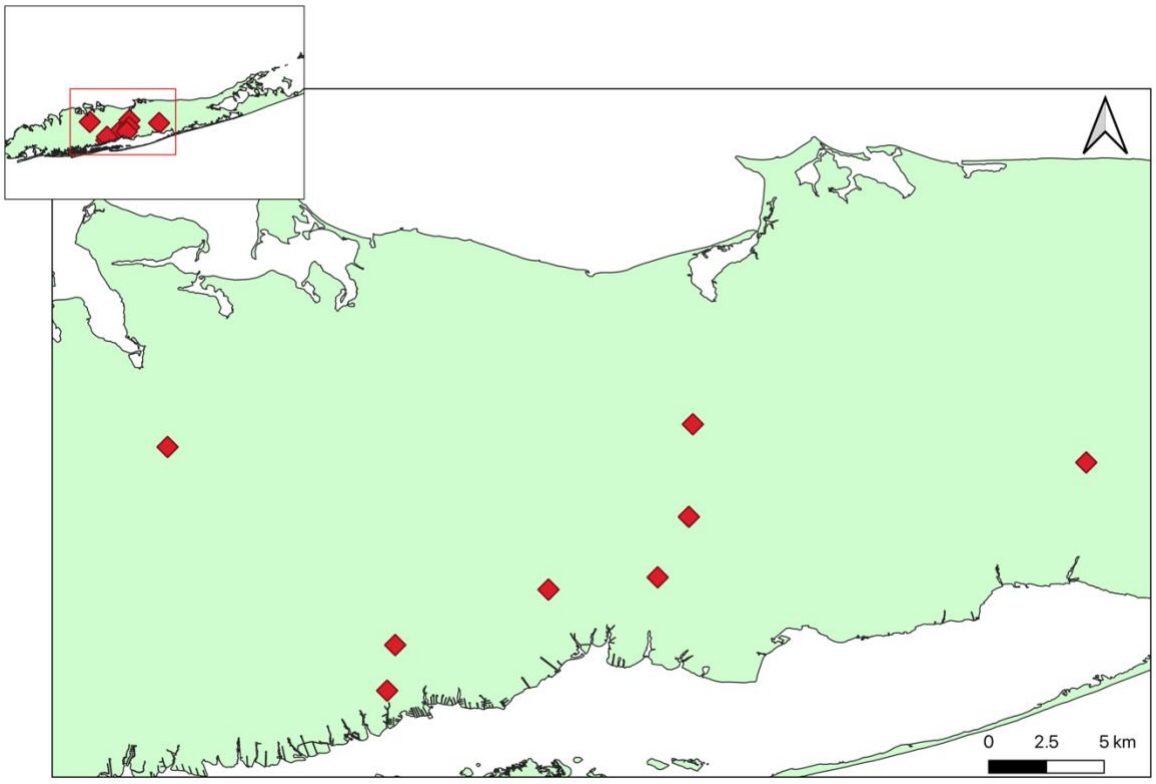

FIGURE 4.1: MAP OF SITE LOCATIONS ON LONG ISLAND, NEW YORK

Red diamonds indicate site locations. Small box in the upper left shows zoomed out map of entire island.

Mosquitoes:

Resting mosquitoes were collected using large custom-designed aspirators (30.5 cm diameter, 114 cm height, 12 V PM DC 2350 RPM, 1/35 Horse power, 3.7 Amp motor) [41] and host seeking mosquitoes that approached collectors were caught with nets. All properties at the eight sites were sampled once per week between 8:00 and 19:00 hrs. The only exception was a small number of individual properties (n=11) where permission to collect was not provided on some

weeks. Two collection teams of three people worked simultaneously at separate properties in residential areas and together at farms. Aspirator collections were conducted by two individuals per property for the length of time necessary to thoroughly sample the entire property, which varied with size and complexity of landscape (most aspirator collection times were between 7-12min; range from 2.5-17min). Host seeking collections were not initially planned, but were included after large numbers of host seeking mosquitoes were observed during initial collection days. The host seeking collections were therefore conducted opportunistically by a third person responsible for specimen labeling and by all three collectors while sorting through aspirator collections after bags were placed in acetone jars for ~3 min. Anesthetized mosquitoes were then separated into microcentrifuge tubes, placed on ice and transported to the laboratory. Mosquitoes were identified to species using published keys [42], sorted by blood meal status, labeled, and stored at -20°C. A small number of the blood-fed mosquitoes (182) were saved for later blood meal analysis and non-blood fed mosquitoes were utilized for sugar analysis. Mosquitoes were transported on dry ice to Cornell University for further processing. To determine body size, one wing was removed from each mosquito, placed on a slide and measured from the axillary incision to base of fringe hairs [43] using a dissecting microscope and software (Olympus SZX9, Olympus DP22 camera, and Olympus cellSens software).

Flower Census

Beginning in mid-July 2018, the number of blooming flowers per morphospecies (up to 100 blooms) was counted on each farm and residential property (n=54). Morphospecies (morphologically distinct species) were identified using the GardenAnswers phone application (Garden Answers LLC., San Diego, CA) [44]. Flower species varied between properties,

including both ornamental and wildflowers consisting of a wide spectrum of different colors, shapes and sizes. However, species-level identifications were not verified by experts and were therefore not analyzed further. Flowers were categorized into groups representing flower presence: absent (0-3 blooms) and present (>3 blooms). A range of zero to three was chosen to represent an absence of flowers rather than zero because three was a natural break point in the data, with only one mosquito collected on a property with 6 or 8 flowers, and all others on properties with at least 9 flowers, creating a natural gap between mosquitoes collected on properties with 0-3 blooms and the rest of the dataset. Properties with up to three flowers had relatively little nectar and were therefore considered an appropriate comparison group to more highly flowered properties, expanding the number of mosquito observations on 'absent' properties by 50% compared with an absolute zero 'absent' group.

Fructose Detection

Cold Anthrone Assay

Fructose concentration was measured using the cold anthrone colorimetric assay [45]. At room temperature, anthrone solution reacts with fructose, but not other sugars. The assay is indicative of plant feeding and does not measure blood sugars (primarily glucose) or stored sugars (trehalose), although non-sugar fed teneral mosquitoes contain small amounts of fructose.

Mosquitoes were homogenized in 1.7 ml microcentrifuge tubes using a lyser (FastPrep-24 Classic Instrument, MP Biomedicals, USA) at 4 m/s for 30 s with 50 µl of 2% sodium sulfate solution and glass beads (3 mm, Thermofisher). Chloroform methanol (1:2) solution (375 µl)

was added to each tube and vortexed for 8 s and centrifuged for 15 min at 200 x g, extracting fructose into supernatant. Tubes were stored at -20°C until fructose quantification, at which time 10 µl of supernatant was transferred to two wells of a 96-well microplate.

To ensure consistency, standards were produced once via serial dilution and stored at 4°C for the duration of analysis. Two replicates of standards (10 µl of 0, 0.05, 0.1 and 0.2 µg /µl D-Fructose (Fisher Chemical, USA) in 25% ethanol) and samples (10 µl) were pipetted individually into wells on each plate. Thereafter, 240 µl anthrone solution (freshly prepared each day, 67.9 µl distilled water, 172.1 µl sulfuric acid, and 0.339 mg anthrone per sample) was added with a multichannel pipette. Samples were incubated at room temperature for 90 min in a chemical hood. The absorbance of light (630 nm) by the reaction of each sample was measured by the microplate reader (800 TS Absorbance Reader, BioTek, VT, USA) and compared against the standard curve to determine fructose concentration. The mean of the two experimental replicates of each sample was used in analyses, except when experimental replicates were dissimilar, the data were discarded or the sample was reanalyzed.

Baseline Mosquito Fructose Concentrations

During the period of adult collection, pupae were collected from containers on a subset of properties and held in the laboratory until eclosion. Post-eclosion, adult *Ae. albopictus* (n= 78 male, 53 female) were held without sugar and frozen within 12 hrs of emergence followed by sugar analysis with the cold anthrone assay. One male and one female outlier were removed using the Median Absolute Deviation. The remaining mosquito data were used to establish a field baseline level of fructose in teneral *Ae. albopictus* [22]. Field-collected adults with fructose

concentrations greater than one standard deviation above the sex-specific mean baseline concentration were considered to be fructose-positive.

Laboratory Digestion Assay

To determine the time window of fructose detection in *Ae. albopictus* post-sugar meal consumption, we conducted an assay of fructose concentration in sugar fed females over digestion time [46]. *Aedes albopictus* (F6 from NY at 23.5°C and F8 from FL at 28°C) were vacuum hatched and provided with a pinch of pulverized fish food (crushed Cichlid Gold fish food pellets; Hikari, Himeji, Japan). One day later, they were separated into trays of 200 larvae with 1L of distilled water and 4 Cichlid Gold fish food pellets. Pupae were transferred to cages and fed 10% sucrose solution between 1 – 3 d post-eclosion. Males and females were removed before, immediately after, and at 24 hr intervals post sugar feeding. Between nine and twenty mosquitoes were removed per day. Fructose concentration was measured as described above. The assumption of constant variance was not met, so mean fructose concentrations were compared with concentration before feeding using the non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparisons test with Benjamini-Hochberg correction (reported as P_{adj}).

Data Analysis of Sugar Feeding Patterns in the Field

Analyses were performed in R (Version 1.1.463) [47]. Average fructose concentration and proportion sugar fed were calculated for all male and female *Ae. albopictus* collected from June to August 2018. Wing measurements were used to standardize fructose concentration by body size, by dividing total concentration by mm wing length. A subset of *Ae. albopictus* for which we

had flower and weather data (those collected between 23 July - 15 August) were included in the models described hereafter.

A Generalized Linear Mixed Model (GLMM; lme4 package) with binomial distribution was employed to determine the impact of measured variables on sugar feeding probability of a captured mosquito [48]. Random effects included town, address nested in town, date, town-date interaction, and address nested in town-date. Fixed effects included capture method (aspirator or net), sex, presence of open flower blooms on property, and saturation deficit. Initially, several weather parameters were evaluated, including minimum, maximum, and average temperature and humidity, as well as saturation deficit [49].

$$\mathrm{SD} = (1 - \frac{\mathrm{RH}}{100})4.9463e^{0.0621\mathrm{T}}$$

All weather parameters had similar explanatory power in the models, so saturation deficit was chosen for the final model because it included both temperature and humidity in a biologically relevant way. Because the sugar was detectable for up to 24 hrs after consumption in our laboratory assessments, the cumulative saturation deficit over that time was determined by summing the saturation deficit over the six most recent time points (a 24 hr interval) prior to collection time for each mosquito. Flower count was included as a binary variable measuring flower presence as described above.

A linear mixed model was employed to evaluate log fructose concentration standardized by wing length using all the fixed and random effects listed above. Only mosquitoes that were sugar fed were included in this analysis to further understand factors influencing the magnitude of sugar feeding.

For both GLMM and linear mixed models, post hoc analyses were conducted by calculating the estimated marginal means of the effects of individual parameters using the emmeans package[50].

Results

Environmental and flower measurements

For the dates July 23 – August 15, 2018, mean ± SD temperature was 24.4 ± 2.79°C (range 15.4°C - 37.1°C). Mean relative humidity was 87.1 ± 12.2 % (range 1%-100%). Floral counts varied by property visit (collection event on a given property): more properties had flowers present (154) during mosquito collections than absent (20). The median number of flowers per property was 110.5.

Mosquitoes

Between June and August 2018, 2,788 *Ae. albopictus* were collected; 1,263 females (45.3%) and 1,525 males (54.7%). Of these, 2,517 (90.3%) were collected resting on vegetation and other surfaces by aspirator and 271 (9.7%) were captured flying around human collectors with nets (241 female and 30 male). Mosquitoes were captured across 8 sites, with 1,097 (39.3%) from the four farms and 1,691 (60.7%) from the four residential areas. Among the subset of mosquitoes that were captured during the floral census (1,970), 1,827 (92.7%) were collected on properties with flowers present and 143 (7.26%) on properties with flowers absent.

Female wings were 2.71 ± 0.27mm (mean ±SD; range: 1.59 – 3.50mm) and male wings were 2.22 ± 0.22mm (range 1.24 – 3.21mm).

Fructose Detection

Field-caught mosquitoes

Among mosquitoes collected from June through August, a high proportion of both male (756/1,525, 49.6%) and female (528/1,263, 41.8%) *Ae. albopictus* were sugar fed. The percent of sugar fed mosquitoes by each variable is displayed in Table 4.1. Among sugar fed mosquitoes, average female fructose concentration was 0.0488 µg/µl and male fructose concentration was 0.0300 µg/µl. To account for differences in body size, fructose concentrations were standardized by wing length for sugar fed females (0.0180 ± 0.0182 µg/(µl*mm)) and males (0.0134 ± 0.0132 µg/(µl*mm)). Average total fructose content was 18.3µg for females and 11.25µg for males.

TABLE 4.1:SUGAR FED STATUS OF FEMALE AND MALE MOSQUITOES BY SITE, HOST SEEKING STATUS, AND FLORAL PRESENCE

	Female			**Male**		
	N (%)			N (%)		
	Sugar	No sugar	Total	Sugar	No sugar	Total
Site type						
Farm	206 (38.4)	330 (61.6)	536	260 (46.3)	301 (53.6)	561
Residential	322 (44.3)	405 (55.7)	727	496 (51.5)	468 (48.5)	964
Host Seeking Status						
Resting	398 (38.9)	624 (61.1)	1,022	738 (49.4)	757 (50.6)	1,495
Host seeking	130 (53.9)	111 (46.1)	241	18 (60.0)	12 (40.0)	30
Flowers*						
Absent	17 (36.2)	30 (63.8)	47	50 (52.1)	46 (47.9)	96
Present	308 (38.5)	492 (61.5)	800	457 (44.5)	570 (55.5)	1,027

Statistical tests of these differences are discussed in GLMM and linear mixed model results below

*Flower presence was only assessed beginning on July 23, so count reflects number collected thereafter

Baseline Fructose Concentration

Mean ± SD fructose concentrations from field collected pupae that eclosed in the laboratory without sugar were 1.42 ± 2.76 ng/µl for females and 0.935 ± 2.09 ng/µl for males. Baseline concentrations (mean fructose concentration +1 SD) were 4.18 ng/µl for females and 3.02 ng/µl for males. All field-captured adult fructose values above this baseline level were considered sugar fed.

Laboratory Digestion Assay

Male and female *Ae. albopictus* digested fructose within 24 hrs after ingestion (Figure 4.2) at both low (23.5°C) and high (28°C) constant temperatures. Compared with fructose levels before sugar feeding (Day 0), fructose was only detectable on Day 1, immediately after feeding (Kruskal-Wallis test with post-hoc Dunn's test; female 23.5°C: P_{adj}=0.0051; male 23.5°C: P_{adj}=0.0004; female 28°C: P=0.0099; male 28°C: P=0.0001). At the next check point, 24 hrs post-feeding (Day 2), and all days thereafter (Days 3-6), fructose concentrations were either not different from (Kruskal-Wallis post-hoc Dunn's, P_{adj}>0.05) or lower (Female 28°C Day 5 : P_{adj}=0.0105 and Day 6: P_{adj}=0.0052) than concentrations before sugar feeding .

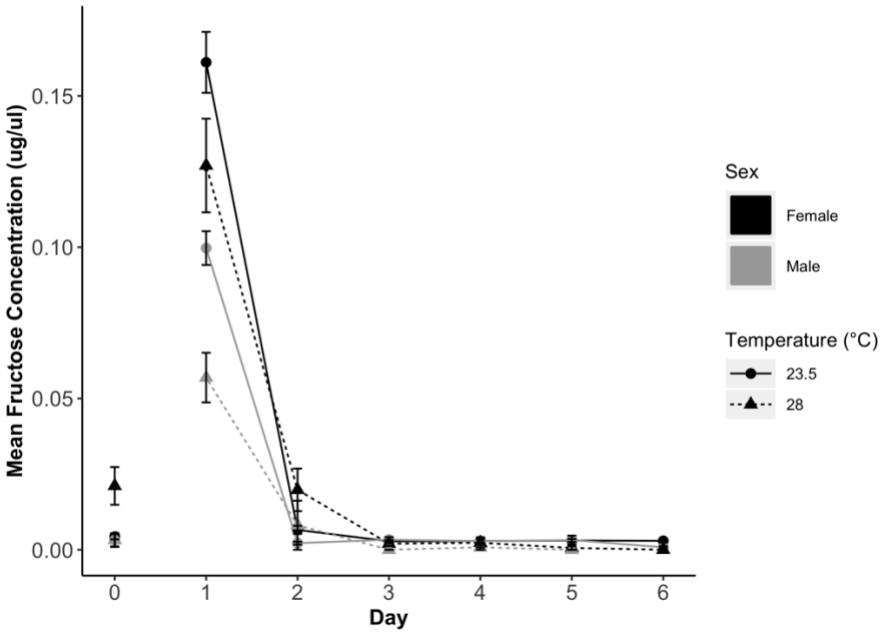

FIGURE 4.2: MALE AND FEMALE DIGESTION OF FRUCTOSE OVER TIME AT 23.5°C AND 28°C

Fructose concentration was measured daily after time of ingestion (Day 1). The daily mean (±SE) fructose concentration is shown for females (black) and males (gray) at 23.5°C (solid line and circle points) and 28°C (dotted line and triangle points). Compared with pre-ingestion fructose concentrations (Day 0), mosquitoes only had significantly higher fructose concentrations on Day 1 (immediately after ingestion) for each sex at each temperature (Kruskal-Wallis Dunn's test; $P_{adj}<0.05$).

Adult Ae. albopictus *sugar feeding patterns*

Effects of environmental and mosquito parameters on sugar feeding status

For the subset of mosquitoes captured after floral and weather data collection was initiated, saturation deficit, host seeking status, and sex influenced the probability of sugar feeding while the number of flowers on a property did not. The likelihood of sugar feeding was affected by dryness as measured by saturation deficit (n=1,970 mosquitoes, β=0.0470, SE=0.0148, *P*=0.00143). More mosquitoes fed on sugar when the saturation deficit was high (i.e. when

weather was hotter and drier) (Figure 4.3). Host seeking mosquitoes (n=151) were more likely to be sugar fed than resting individuals (n=1,673; β=0.527, SE=0.201, *P*=0.00870). Males (n=1,042) were more likely to be sugar fed compared with females (n=782; β=0.394, SE=0.110, *P*=0.000321). The relative abundance of flowers did not affect the likelihood of sugar feeding; mosquitoes collected on properties with flowers (n=1,827) were not more likely to be sugar fed than those captured on properties with no flowers (n=143; β= 0.0728, SE=0.290, *P*=0.802).

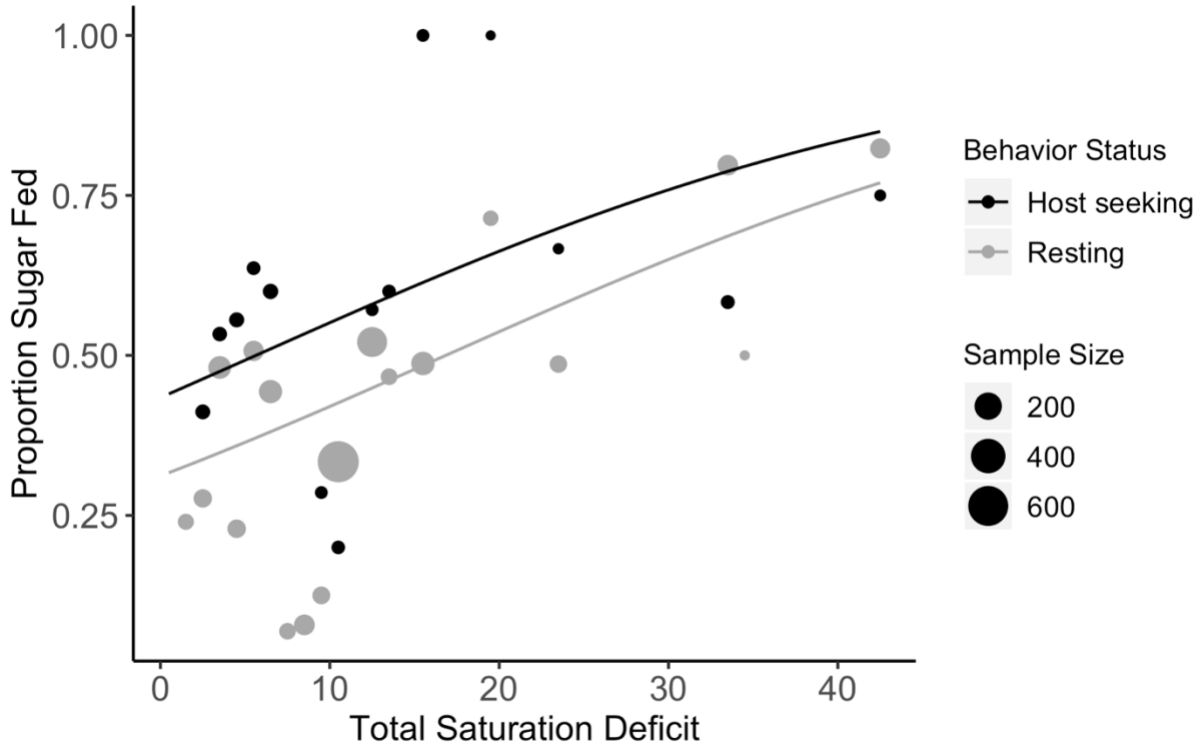

FIGURE 4.3: THE PROPORTION OF SUGAR FED MOSQUITOES BY SATURATION DEFICIT FOR HOST SEEKING (BLACK) AND RESTING (GRAY) MOSQUITOES

Mosquitoes were grouped by 1 unit of saturation deficit. The total number of mosquitoes collected per unit saturation deficit is represented by point size. The predicted probability of sugar feeding by saturation deficit is indicated by the lines. As saturation deficit increased, the likelihood of capturing a sugar-fed mosquito increased (GLMM, *P*=0.00143). Mosquitoes captured while host seeking were more likely to be sugar fed than while resting (GLMM, *P*=0.00870) [50].

Effects of environmental and mosquito parameters on fructose concentration ingested

The linear mixed model results showed that the fructose concentration in sugar fed mosquitoes was predicted by flower abundance but not by saturation deficit, sex, or host seeking status. Among sugar fed mosquitoes (n=832), those collected on properties with flowers present (n=765) had significantly higher fructose concentration per mm wing length than those collected on properties with flowers absent (n=67) (β=0.325, SE=0.142, *P*=0.0253) (Figure 4.4). Males (n=507) took marginally smaller sugar meals compared with females (n=325) even when controlling for body size differences between the sexes (β=-0.133, SE=0.0691, *P*=0.0553). There was no significant effect of host seeking status (host seeking vs resting, β=0.217, SE=0.116, *P*=0.0611) or saturation deficit (β=0.00844, SE=0.00711, *P*=0.253) on fructose concentration per mm wing length.

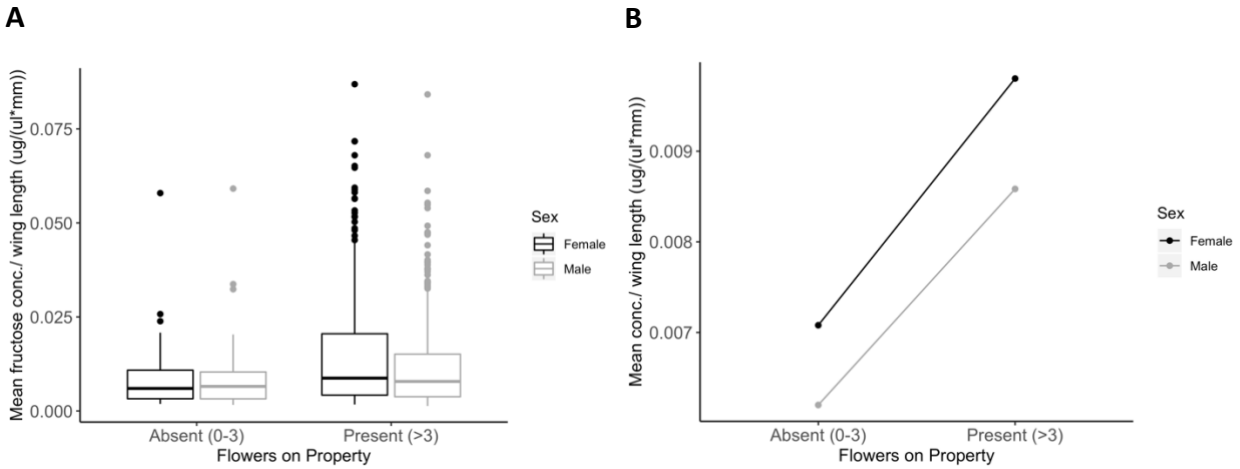

FIGURE 4.4: FLOWER PRESENCE AND FRUCTOSE CONCENTRATION

A. Mean fructose concentration standardized by wing length for female (black) and male (gray) *Ae. albopictus* on properties with and without flowers. Points show individual mosquito fructose concentration standardized by wing length of outliers. Includes both resting and host seeking mosquitoes. **B. Predicted fructose concentration by flower presence and sex**. Mosquitoes collected on properties with flowers present

(LMM, P=0.0253) had higher fructose concentration per mm wing length than those collected on properties with flowers absent. Females had marginally higher fructose concentration compared with males (LMM, P=0.0553).

Discussion

Sugar feeding patterns of field captured *Ae. albopictus* mosquitoes have only been reported from three other locations: Israel, Italy, and Florida [17, 24, 33]. Our study reports Asian tiger mosquito sugar feeding patterns for the first time from the northern edge of its invasion in the Eastern USA. Using robust sample sizes, we demonstrated that a large proportion of both male and female *Ae. albopictus* fed on plant or homopteran derived sugar sources within 24 hrs of capture. Our results suggest that sugar feeding behavior increases when environmental conditions are dry and may vary by behavioral status (host seeking vs resting). Furthermore, mosquitoes collected on properties with flowers had higher fructose concentrations compared with those collected from properties with no flowers.

A large percentage of males (49.6%) and females (41.8%) collected from our field sites were sugar fed. Our laboratory assays demonstrated that *Ae. albopictus* digest fructose within 24 hrs of consuming a sugar meal at 23.5 and 28°C. According to this window of detection, and considering the average field temperatures during collections, approximately half of the field-captured mosquitoes fed on sugar daily. Sugar feeding estimates may be influenced by the concentration and composition of sugar consumed, which varies between flower species' nectar and between alternative sources of sugar. In our study we used a 10%-sucrose solution representing the low end of sugar concentrations in nectar (7-70%) and only one of the constituent sugars, consistent with prior sugar digestion studies [46, 51]. Sucrose is a disaccharide, containing a glucose and fructose moiety (two other common nectar sugars);

disaccharides are known to react in a similar manner to their monosaccharide constituents in the cold anthrone assay, reducing potential sources of variation [52]. However, it is possible that *Ae. albopictus* digestion rate of the sugar source we tested with laboratory mosquitoes may not be representative of all available natural sugar sources. In a temperate region of Italy, similar rates of sugar feeding were detected among released males (48% at 72 hours post-release) compared with wild males in our study [17]. In the arid climate of Israel, sugar feeding tended to be more common; the percentage of sugar fed mosquitoes ranged from 41.3% to 74.1% based on season and site [24].

In Long Island, *Ae. albopictus* that experienced higher saturation deficits (hotter, drier weather) during the 24 hours prior to collection were more likely to contain a sugar meal than those collected during lower saturation deficits. Bellini et al. (2014) observed a similar pattern with field-released males when assessing sugar feeding devices; the percentage of sugar positive males was correlated negatively with relative humidity and positively with temperature at control sites [17]. It is possible that high saturation deficit leads to dehydration and ultimately triggers higher rates of sugar feeding, especially on more dilute sources. Maintaining water balance is essential for insect survival [53, 54] and others have described insect foraging behaviors that balance physiological needs for water and sugar through choice of nectar dilution levels [55-58]. Working with mosquitoes, Hagan et al. (2018) found that blood feeding was prompted by dehydration [27]. Although sugar and blood feeding are different behaviors and dilute nectars can contain similar or lower levels of water compared to blood, it is possible that mosquitoes use the same set of physiological cues to prompt sugar feeding under dehydrating conditions. Upshur et al. (2019) demonstrated that sugar feeding increased between 20°C and 30°C, further

suggesting the impact of environmental conditions on the tendency of mosquitoes to ingest sugar [59].

In our study, the presence of flowers did not influence the likelihood of *Ae. albopictus* sugar feeding but did impact the amount of sugar ingested when they did feed. Residential property sizes varied in our study but tended to be small and within the flight range of *Ae. albopictus* [60, 61], so it is possible that sugar fed mosquitoes collected in yards without flowers originally sugar fed in adjacent yards with greater floral abundance, and subsequently used some of the fructose in flight. This could explain why we observed consistent likelihood to sugar feed between flower categories, but different fructose concentrations between mosquitoes collected on properties with and without flowers. Alternatively, sugar fed mosquitoes in yards without flowers may have consumed non-nectar sources, such as honeydew or plant tissue. Parasitoid wasps fed on honeydew had lower fructose levels compared with those fed on nectar [62] and plant leaves generally have lower concentration of sugars than nectar [63]. This would also account for the equal likelihood of feeding and different fructose concentrations by flower presence.

Only one other published study has investigated floral abundance and *Ae. albopictus* sugar feeding and differs from our results. In Israel,under arid environmental conditions, Müller et al. (2010) found a difference in sugar feeding likelihood by flower abundance: 42% and 68% of females from low and high floral abundance sites, respectively, contained sugar [24]. It is difficult to compare the two studies due to substantial differences in environmental conditions. Houses without flowers in Long Island, USA still had significant vegetation and potential non-nectar sugar availability, in contrast to the less vegetated “dry wasteland” site in Israel. Sampling limitations in our study restricted floral surveys to properties where mosquitoes were collected,

preventing inclusion of flowers in neighboring yards within the flight range of *Ae. albopictus*. Quality of floral resource was also not considered, such as nectar quantity or quality, which can be highly variable [64]. The design of the floral surveys also prevented analysis of floral density or species effects on sugar feeding.

A subset of host seeking mosquitoes were opportunistically captured with nets as they flew around human collectors. These mosquitoes were more likely to contain sugar meals than those collected with aspirators while resting on vegetation and other surfaces. While some studies have reported reduced blood feeding after sugar feeding [8, 10, 11], it is possible that teneral females seek sugar meals shortly after eclosion before blood feeding [9], explaining higher sugar content in host seeking females. This observation warrants further, more systematic investigation. In addition, it highlights the importance of considering collection method biases when assessing sugar feeding prevalence and should be an important consideration when designing and analyzing sugar feeding study results.

These sugar feeding patterns will likely influence the success of sugar-based control techniques, such as ATSBs. While this control strategy has only been assessed for *Ae. albopictus* populations in Florida and Israel [21, 35-39], our results provide insight into the potential for deployment of ATSBs in our study region. In Israel, 62.7% of female *Ae. albopictus* were sugar fed at a natural garden site; meanwhile, ATSB deployment reduced biting pressure by 85% at another site under similar conditions [24, 37]. The comparatively lower percentage of sugar fed females in Long Island (41.8%) may result in weaker reductions in biting pressure in our study region, but the sugar feeding rates were nevertheless sufficient to warrant further investigation of ATSB-based control methods in Northeastern US. Our results suggest that control success in our region may be maximized if ATSBs are deployed during hot, dry conditions and in locations

with fewer flowers and less competition. Furthermore, the tendency of *Ae. albopictus* to sugar feed prior to blood feeding may increase the public health impact of ATSBs by concentrating control pressure before the point of pathogen acquisition or transmission.

While relatively little is known about *Ae. albopictus* sugar feeding, this behavior has been studied in other mosquito species, including *Ae. aegypti*, which shares some ecological similarity. In Thailand, the percentage of sugar fed *Ae. aegypti* females increased in the dry season (16%) compared with the rainy season (5%), potentially echoing the effect of saturation deficit on proclivity to feed found in our study [22]. Other studies have also shown remarkably low levels of sugar feeding for female *Ae. aegypti [28, 65]*. However, *Ae. aegypti* females in Texas had higher rates of sugar feeding (47.91%), similar to what we report for *Ae. albopictus* in our current study [66]. Another important vector species, *Anopheles gambiae*, had low rates of sugar feeding in Kenya; the percentage was higher for host seeking (14.4%) females compared with resting (6.3%), similar to the trend found in our study [67]. However, recent studies suggest that *An. gambiae* may feed on sugar more often than originally thought; populations can be successfully controlled by ATSBs [68], survival is reduced by removal of a flowering invasive shrub [30], and both males and females are robustly attracted to a number of different plants [69].

As the ability to detect DNA from mosquito plant meals improves, future studies could explore sugar feeding with greater resolution than the cold anthrone test affords. Next-generation sequencing has been employed to successfully identify plant meals of mosquitoes and other blood feeding Diptera [70, 71]. Additional studies of *Ae. albopictus* plant meal origin would be beneficial in ATSB lure design optimization. However, results of these analyses must be

interpreted with caution as they may bias towards non-nectar sugar sources that are more likely to be detected via DNA-based analyses due to minimal DNA content of nectar.

Our results demonstrate, for the first time, sugar feeding patterns by temperate populations of *Ae. albopictus* in the United States. This is only the fourth field study on this important mosquito behavior and provides us with insights into conditions that might influence sugar feeding variation, including saturation deficit, flower presence, and host seeking. In light of the high frequency of sugar feeding in the study population, our results show promise for deployment of attractive toxic sugar baits for *Ae. albopictus* control in the region and provide insight into potential modifications of bait timing and placement to maximize success.

Data Availability: Data will be deposited in Cornell University Library's institutional repository, eCommons (https://ecommons.cornell.edu), for preservation and access. Datasets will be available via the world wide web without restriction. eCommons provides each item with a persistent identifier and is committed to preserving the binary form of the digital object. The data can be accessed at this DOI: https://doi.org/10.7298/kfnn-3296.

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RESEARCH SUMMARY AND FUTURE DIRECTIONS

Research Summary

Together, the chapters of my dissertation provide a holistic view of *Ae. albopictus* feeding biology, integrating across ecology, physiology, and behavior to measure blood and sugar feeding patterns, fitness, and host preference. It is especially important to address these under-studied questions for *Ae. albopictus* due to its expansive distribution and robust vector competence for many pathogens.

In chapter one, I laid the foundation of my dissertation. I provided a detailed explanation of the importance of accurately assessing and interpreting blood feeding patterns and host preference. I also provided guidelines for future researchers preparing to conduct studies in this field, including how to avoid certain forms of bias and the importance of acknowledging them when they cannot be avoided. The case study on *Ae. albopictus* demonstrated the importance of accurate interpretation. Previously published *Ae. albopictus* feeding pattern studies show a wide range of host usage, both in terms of the level of anthropophagy (human feeding) and the number of host species. Most of these studies are not paired with host availability measures, so it is impossible to determine the contribution of this important environmental factor to the distinct feeding patterns. As the case study demonstrates, the tendency of some to ascribe feeding patterns to host preference has led *Ae. albopictus* to be considered both anthropophilic (human preferring) and generalist in the literature. There has been little direct assessment of host preference to clarify between these conflicting descriptors. The studies that have been conducted have suggested that the species is anthropophilic, but these results may have been influenced by host defenses and other factors, and also do not include populations that have been found to have

lower levels of anthropophagy. Chapters two and three of my dissertation sought to fill these gaps.

For the feeding pattern study in chapter two, I presented a blood meal analysis of *Ae. albopictus* collected in Long Island, New York, near its invasive edge in the Northeastern United States. Ninety blood meals were identified, pertaining to ten host species, with higher host diversity and lower anthropophagy at farms compared with residential areas. Weekly household interviews regarding human and pet abundance and time outdoors were used to calculate host feeding indices (HFIs), a measure of host usage relative to availability. Both abundance and time-weighted HFIs indicated over-utilization of cats and dogs compared with humans. Camera traps were used to estimate the abundance of wild animals and domestic cats and calculate forage ratios, another form of feeding metric. Forage ratios suggested over-utilization of cat and opossum and under-utilization of raccoon, bird, and squirrel.

These feeding patterns were in stark contrast to a recently published blood meal analysis in nearby Baltimore, Maryland, where *Ae. albopictus* fed predominantly on rat blood. We therefore wanted to understand if differential fitness following feeding on different host species blood was a driver of feeding patterns through an evolutionary pressure to preferentially feed on the host eliciting the highest fitness. In the second part of chapter two, we fed New York *Ae. albopictus* blood from human, horse, cat, rat and opossum and measured survival and fecundity. We then compared these fitness parameters to Baltimore *Ae. albopictus* fed human and rat blood. The results did not show major differences in mosquito fitness by host species, which suggests that fitness does not drive feeding patterns in the Northeastern US.

However, measurement of fitness parameters is also not a direct assessment of host preference, it simply investigates one route by which host preference can evolve. We therefore

could not rule out that *Ae. albopictus* has innate host preferences. Furthermore, while Long Island and Baltimore feeding patterns are different from one another, they do not represent opposite ends of the anthropophagy spectrum observed worldwide. When grouped by anthropophagy level, in fact, Long Island and Baltimore group together on the lower end of human usage.

In the third chapter, we directly assessed the host odor preference of *Ae. albopictus*. Using a dual-port olfactometer, we compared three low anthropophagy and three high anthropophagy populations to test the hypothesis that distinct host preferences drive divergent feeding patterns around the world. No differences were observed in the probability of choosing human between the *Ae. albopictus* populations, suggesting that there is limited variation for this trait and that observed differences in feeding patterns are more likely the result of differences in other factors, such as host availability. We also compared these six *Ae. albopictus* populations to previously characterized zoophilic and anthropophilic *Ae. aegypti* colonies. We found that *Ae. albopictus* was less likely to choose human compared with the anthropophilic, globally invasive lineage of *Ae. aegypti* and behaved similarly to the zoophilic *Ae. aegypti* from Uganda. This study provides the first direct comparison of the host preference of these important vectors with overlapping ecologies and pathogen associations.

The fourth and final chapter of my dissertation investigated a distinct, yet complimentary aspect of *Ae. albopictus* feeding biology – sugar feeding. We used the cold anthrone assay to determine the proportion of Long Island, NY *Ae. albopictus* males and females that were sugar fed. We then assessed the impact of several environmental and mosquito parameters on both the likelihood of being sugar fed and the concentration of fructose. We found approximately half of males and 41.8% of females were sugar fed. Our findings suggest that *Ae. albopictus* were more

likely to sugar feed when the environment was hot and dry. Comparing mosquitoes by host-seeking status indicated that host-seeking mosquitoes were more likely to be sugar fed than their resting counterparts. Lastly, among sugar fed mosquitoes, those captured on properties with flowers (>3 blooms) had higher fructose concentrations than those captured on properties without flowers (0-3 blooms). This was the first analysis of *Ae. albopictus* sugar feeding in the Northeastern United States and provided contextual details that can inform decisions regarding sugar-based control strategies.

The four chapters of my dissertation each approach the feeding biology of *Ae. albopictus* from a different angle. Mosquito feeding biology is complex and poorly understood, with a variety of factors that can influence the outcomes, from the environment to the physiological needs of the mosquito. By examining *Ae. albopictus* feeding biology from each of the distinct angles described in my dissertation, we can begin combining these pieces of evidence to create a better-informed narrative about this important vector species. A combination of controlled laboratory experiments and field studies that account for environmental parameters complement one another and reveal the underlying drivers of patterns and how they interact in a complex world.

Future Directions

How often do mosquitoes approach a host and decide not to bite – and why?

Host seeking is an energy-intensive activity. Yet, we know that mosquitoes can decide to abandon their host seeking attempts before biting as they approach the host and integrate across additional shorter range host seeking cues. To better understand the feeding decision process, it would be helpful to understand how often mosquitoes decide not to bite after beginning an

approach. It would be especially important to understand which cues result in the abandonment of host seeking – this information may provide the key to new biting deterrent strategies.

Have specialist mosquito species evolved behavior to evade host defenses particular to the preferred host? Are they able to better survive their preferred host's defenses than generalist mosquitoes?

The evolution of specialization can come with both advantages and disadvantages. In terms of mosquito host seeking, specialization narrows the host range of the mosquito, potentially reducing the number of blood meal opportunities. To compensate for this loss, specialization must provide some advantages. For select species, there is evidence of several advantages, including increased fitness. Another possibility is that host preference specialization also allows for the specialization of host defense evasion. Do specialist mosquitoes have specific host defense evasion behaviors that allow them to improve survival and biting success rate on their preferred host compared with generalist mosquitoes? Does this coincide with decreased success on other non-preferred hosts?

How do mosquitoes balance sugar and blood feeding throughout their lives?

Female mosquitoes feed on both blood and sugar for different aspects of their nutrient needs. How do they balance these two feeding needs throughout their lives? Relatively little is known about the impact of sugar feeding on blood feeding and vice versa for mosquitoes in the field, which experience very different energy needs than those in the laboratory. Are there certain times of life when mosquitoes are more likely to seek one food source compared with the other? Does acquiring a sugar meal prior to host seeking impact host seeking success? Do mosquitoes

avoid host seeking for some period of time after sugar feeding? All of these questions and more can help us to understand how female mosquitoes balance these two disparate food sources.

Is next generation sequencing an unbiased method for assessing sugar sources? Which plant species do Ae. albopictus *feed on?*

While measuring sugar feeding with the cold anthrone assay is straight-forward, it does not provide any information about the sugar source. Some researchers have used next-generation sequencing to identify the sugar source, however it is not clear whether this provides a biased assessment due to unequal amplification based on DNA concentration in the source. Most nectar is DNA-poor, so nectar sources may be under-represented compared with plant tissue sources. The presence and extent of bias of next-generation sequencing techniques for identifying sugar sources should be evaluated. This or other techniques should be developed to determine the source of *Ae. albopictus* sugar feeding, which can be used to improve the design of ATSB lures.

Is sugar a limiting resource for Ae. albopictus *in certain environments?*

In our study, we found that the likelihood that an *Ae. albopictus* mosquito was sugar fed was not different between properties with and without flowers. However, among those that were sugar fed, mosquitoes on properties without flowers had a lower concentration of fructose compared with those on properties with flowers. These results could stem from several scenarios. For example, sugar fed mosquitoes on properties without flowers may have flown from properties with more flowers. Or, sugar fed mosquitoes on properties without flowers may have fed on plant tissue, which may result in lower fructose concentration. It is difficult to tease these and other possibilities apart because the properties we sampled were relatively small and directly

bordered one another. It would be interesting to determine the impact of flower presence/abundance on the probability of sugar feeding in locations where differences in flower presence exist at a wider scale. If differences do exist, is sugar a limiting resource at those locations?

Is Ae. albopictus *anthropophilic or generalist?*

In my opinion, there is still no definitive conclusion as to whether *Ae. albopictus* is anthropophilic or generalist. The dual-port olfactometer experiment described here provides good evidence that *Ae. albopictus* is less anthropophilic than *Ae. aegypti*. The results also show that *Ae. albopictus* is less likely to choose human than guinea pig. However, the experiment was conducted using only one part of the human body and only compared against one non-human animal, so it does not prove whether *Ae. albopictus* would choose guinea pig (or other animals) over human in the field. Only two experiments have been conducted on *Ae. albopictus* host preference in the field, both with potential sources of bias. More experiments assessing *Ae. albopictus* host preference should be conducted using different types of experimental design in the lab and field to arrive at a consensus conclusion.

Beyond the impact on vector density, do some control methods, such as indoor residual spraying, further reduce the vectorial capacity of mosquitoes to transmit anthroponotic diseases through reduction in human feeding?

It is very difficult to directly assess the impact of control on disease transmission, especially for diseases that occur at a low frequency. As a result, vector control methods are often assessed for efficacy based solely on vector density measures. In addition to an impact on vector density, it is

plausible that certain control methods may impact feeding patterns, for example by forcing mosquitoes to feed outside of houses where they might encounter non-human host species at higher rates than inside houses. This could be assessed by conducting a blood meal analysis alongside other measurements in treatment and control sites to compare feeding patterns. If there is an impact of control treatment on feeding patterns, the effect of this on disease transmission could be predicted through entomological inoculation rate models.