THE TRANSMISSION EFFICIENCY OF *PLASMODIUM YOELII* INFECTED MOSQUITOES

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland

April, 2015

ABSTRACT

Malaria is a life-threatening infectious disease caused by the *Plasmodium* parasite. Nearly half of the global population is at risk of acquiring malaria and there are approximately 500,000 deaths and 200 million cases annually. The infective form of the parasite, the sporozoite, is transmitted by the female *Anopheles* mosquito as she probes on a human host in search of a blood meal. Although it has been over 100 years since Ronald Ross discovered that *Anopheles* mosquitoes are the vector for the parasite, we still do not fully understand the early transmission dynamics of *Plasmodium*. One aspect that is poorly described is the probability of developing a blood stage infection after the bite of an infected mosquito. The entomological inoculation rate estimates the number of infected bites that an individual receives, but at present there is no understanding of the likelihood that sporozoites inoculated by a bite will successfully infect the host. This work provides the first laboratory estimate of the proportion of infected bites to a naïve host that result in a blood stage infection. In addition, four factors that may influence the transmission efficiency—the intensity of salivary gland infection, the duration of probing, the anatomical location on the host exposed to the mosquito bite, and the success of the mosquito in acquiring a blood meal—are considered. Using the rodent parasite *Plasmodium yoelii* in *Anopheles stephensi* mosquitoes, we determined that the transmission efficiency of a single mosquito bite is 21%. Further, the proportion of bites that result in an infection is not dependent on probe time, probe location, or acquisition of a blood meal; however a significantly greater probability of blood stage infection is present when the salivary glands of the probing mosquito are heavily infected.

ACKNOWLEGEMENTS

When I was accepted into Hopkins to pursue a graduate degree, I was regularly announcing that I was, finally, “going to become a scientist.” This dream had been a long time in the making, and of the many people that contributed its realization, my thesis mentor— Dr. Photini Sinnis— has without a doubt had the greatest impact on my development into the scientist that I was so eager to become. It is through her expertise, support, and guidance over the past year and a half that I have been able to complete this thesis research and grow as a scientist, and for that I am sincerely grateful.

Many other people were involved in this research and the production of this thesis, without which this work would not have been possible. I’d like to thank Dr. Fidel Zavala for serving as my second thesis reader, and for his valuable insights into the statistical approaches in my analysis. I would also like to express appreciation to Dr. Douglass Norris, for frequent discussions on the ecological aspects of malaria transmission; to Dr. David Sullivan, for giving me the opportunity to invade his parasitology class with my project and for his continued mentorship; to Gail O’Connor, for ushering us into MMI and back out again; and to the many students and faculty in the department that provided support and feedback during my time here. I would especially like to acknowledge Godfree Mlambo and Chris Kizito for providing the mosquitoes and parasites with which my experiments were conducted; without their expertise, my research would not have been possible.

I have been fortunate enough to meet and work with many wonderful people during my time at Hopkins, but the camaraderie, support, guidance, and friendship of Amanda Balaban, Christine Hopp, and Daniel Ragheb has been immeasurable. Thank you for welcoming me into your weird little corner of the world, and for the help and encouragement along the way. I would not want to tear the heads off hundreds of mosquitoes alongside anyone else. I have also appreciated the company of the many other permanent and temporary members of the Sinnis Lab: Lirong Shi, Jianyang Wang, Renuka Joseph, Hannah MacLeod, and Steven Wellard.

These two years would not have been possible without the endless support of the many friends I have picked up along the way. Thanks to the other members of my cohort, especially Maureen Kessler and Jean Clement, who have made this journey all the more fun; to my roommates, Kaley Lambden, Hannah Macleod, and Maureen Kessler for keeping me sane despite it all, and for all the days you started the morning coffee; and a heartfelt thanks to Maria Cristina Bravo for your guidance during the early stages of my mission to become a scientist, and for providing much-needed support during my first year at Hopkins.

Finally, the unending support of my family has been very important over the years. To Hannah and Rayna, thank you for listening to me talk about mice and mosquitoes, and for your ideas and encouragement from day one, literally. And thank you to my parents, Barry and Martina: nearly 20 years ago, my parents schlepped me and my two sisters to Belize for a month, as part of a medical resident exchange

program. It was there that I was first exposed to malaria, albeit protected from its destruction by chloroquine tablets, and the curiosity that was piqued on that trip has only grown in the decades since. I don't think I have ever properly thanked you for the audacity to bring three kids under the age of ten to a foreign, developing country, and for the impact that the adventure had on my trajectory as a scientist. Your support as I have navigated the early part of my life and career has been monumental, unwavering, and is greatly appreciated.

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INTRODUCTION

Malaria is an infectious disease caused by invasion of erythrocytes by a single-celled protozoan of the genus *Plasmodium.* Once widespread throughout most of the world, malaria is now primarily a disease of the tropics and subtropics, where it is responsible for approximately 200 million cases and 500,000 deaths annually [1]; the majority of these deaths occur in young children in sub-Saharan Africa. The disease is named after the belief that it was spread by 'bad air', or mal'aria, a term first coined in the 18th century [2] that remains today, despite great advances in the understanding of the disease and it's transmission. The causative agent and vector of malaria were defined in the late 19th century [3] and the past 100 years have seen tremendous public health efforts successfully reduce proportion of the globe in which transmission occurs, as well as the morbidity and mortality associated with malaria [4]. Despite these efforts, malaria remains one of the leading causes of death in children worldwide.

Plasmodium Lifecycle

The *Plasmodium* genus belongs to the phylum Apicomplexa, a group of protists characterized by a conserved apical complex and subpellicular arrangement of microtubules [5]. Many other disease-causing protists are in this phylum, including *Babesia, Cryptosporidium,* and *Toxoplasma*, all of which have complex lifecycles involving both sexual and asexual reproduction. The definitive host of *Plasmodium*—the host in which sexual reproduction occurs—is the female *Anopheles* mosquito [6]. When taking a blood meal from an infected human, the mosquito ingests the sexual stage of

the parasite, the gametocytes, which are circulating in the blood of the human host [6]. The drop in temperature and other external cues cause the gametocytes to mature into gametes [7]. The male and female gametes fuse in the midgut of the mosquito to form an ookinete; this fertilized zygote is motile, and traverses the midgut wall of the mosquito to form the oocyst, where sporozoites are produced in large numbers [6]. The sporozoite is the infectious stage of the parasite, and takes on an elongated form that is 10 µm in length, and approximately 1 µm in diameter [8]. When the sporozoites are mature, they are released into the hemocoel and circulate in the hemolymph until contacting salivary glands, which the sporozoites must invade prior to infecting the next host; approximately 15% of circulating sporozoites will successfully invade the salivary glands [9]. *Plasmodium* transmission takes place when salivary gland sporozoites are injected into the skin of a mammalian host as the mosquito probes for blood [10]. Once in the skin, some sporozoites find and invade blood vessels [11], entering the circulation, where they are carried by the blood flow to the liver. Here sporozoites are arrested, cross the sinusoid into the liver parenchyma, and invade hepatocytes where they develop into an exoerythrocytic form containing thousands of hepatic merozoites [12]. When the merozoites are released from the hepatocyte, they invade erythrocytes and initiate the blood stage of infection. In the red blood cell they grow to trophozoites, which replicate by schizogony to produce 10-14 merozoites and are released as the red blood cell bursts [6]. These merozoites can then invade new erythrocytes. The cyclic fevers that characterize malaria symptoms coincide with the release of schizonts from the red blood cells, and occur at varying intervals depending on the length of the

parasite's lifecycle in the red cell, which is specific to each *Plasmodium* species [13]. Some merozoites develop into gametocytes, the sexual stages of the parasite that, when taken up by the mosquito as she obtains a blood meal, complete the cycle of transmission [6].

There are five species of *Plasmodium* that infect humans. *P. falciparum* causes the most severe human form of the disease, which may be due to the ability of the blood stage parasites to cytoadhere to the vasculature and thus avoid splenic disruption [10]. *P. falciparum* is also able and to invade red cells of all ages [14], and together these features allow *P. falciparum* to attain much higher parasite densities in the host than other species. Blockages and/or inflammation due to the parasite can cause severe pathology in various organs [6]. The most fatal outcome of malaria is pathology in the brain, which leads to cerebral malaria and can result in death [15]. Infection of the placenta can also cause severe outcomes for the mother and fetus [6]. Pharmaceutical prophylaxis and treatment are effective against *P. falciparum* [6], however the increasing challenge of drug resistance combined with limitations in healthcare infrastructure result in an ongoing malaria burden throughout most of sub-Saharan Africa.

The other species of *Plasmodium* that are cause malaria in humans are *P. vivax*, *P. malariae*, and *P. ovale.* Recently, the primate malaria parasite *P. knowlesi* has also been found in humans [16] and infection with this species can also cause severe symptoms. *P. vivax* and *P. ovale* present the additional complication of relapses, due to

persistent liver forms that can lie dormant for months to years [6], which make effective treatment difficult.

The route of the sporozoites during transmission

At the interface of the transmission of the parasite from mosquito to mammalian host is the sporozoite. Prior to being injected into the dermis of the host by the probing mosquito, sporozoites are found in her salivary glands. The female *Anopheles* mosquito has two salivary glands, each having three lobes, with salivary ducts that run down the lobes and join into the common salivary duct [17]. This common duct extends down the proboscis of the mosquito, and it is through the common duct that the sporozoites are ejected from the mosquito with during probing (Figure 1). It is believed that only sporozoites in the distal region of the salivary glands have access to the salivary duct and thus are available for transmission; because the proximal regions of the salivary ducts are only about 1 μM wide and heavily sclerosed, sporozoites that are in the glands that abut these regions are unable to enter the ducts [18]. Due to the physical constraints of the duct itself, which is only slightly wider than a single sporozoite [18, 19], the sporozoites are thought to line up single file [20], which may limit the size of the inoculum during the probing event. Thus, despite high numbers of sporozoites in the salivary glands, the proportion of sporozoites that is immediately available for transmission is likely to be small, as demonstrated by the small numbers of sporozoites inoculated by the probing mosquito [21]. While the effect of these limitations is not completely defined, it has been shown that the number of sporozoites that are found in

the salivary duct is low [22], and the ejection of sporozoites takes place predominantly in the first three minutes of probing despite salivation extending for as long as eight minutes [22]. However, the experiments that led to these conclusions were performed with artificially salivating mosquitoes, not with mosquitoes probing on live animals while searching for blood, and thus do not fully describe what takes place during probing.

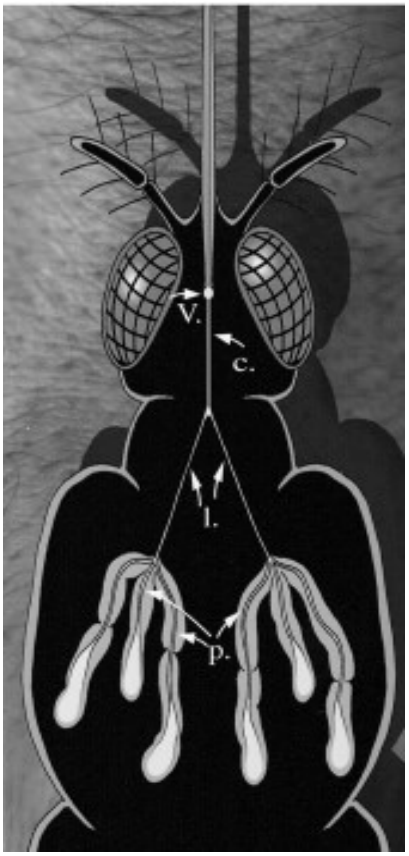

Figure 1. A schematic representation of the female mosquito salivary gland system. The two salivary glands, with three lobes each, extend into the body cavity of the mosquito. The salivary ducts are indicated by arrows; primary (p), lateral (l), and common (c) salivary ducts are shown. The salivary valve (V) is located between the common duct and the salivary canal of the proboscis, and creates a temporal separation between probing/salivating and imbibing blood.

Image credit: Frischknecht F, Baldacci P, Martin B, et al. Imaging movement of malaria parasites during transmission by *Anopheles* mosquitoes. *Cell Microbiol*. 2004;6(7):687-694.

After invading the salivary glands, the sporozoites are transmitted to the mammalian host in the mosquito's saliva. When probing a host in search of a blood vessel, the mosquito ejects saliva containing anticoagulants and anti-inflammatory molecules [17] which allow her to successfully acquire a blood meal. The salivary canal

of the mosquito is distinct from the food canal [17] and is controlled by a valve [22]; this valve results in a temporal separation of the saliva inoculation associated with blood-seeking behavior (probing), and the sucking of blood during feeding. Figure 2 illustrates the mosquito mouthpart architecture. Because the sporozoites are found in the saliva of the female *Anopheles* mosquito, transmission is only possible during probing behavior; once the mosquito has located a blood vessel and begins feeding, the switch from spitting to sucking prevents further inoculation of saliva [17] and thus sporozoites.

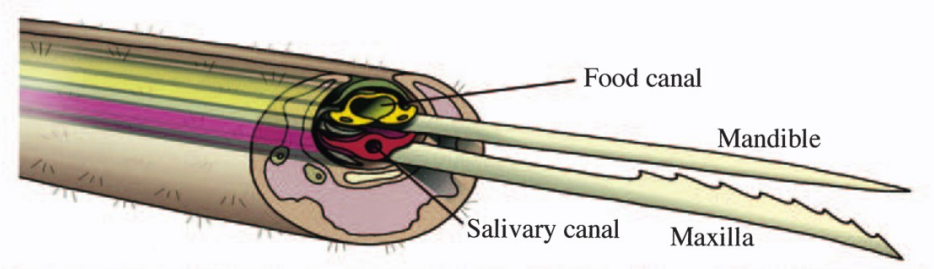

Figure 2. Schematic representation of the mosquito proboscis. The hypopharynx (red) contains the 3 µm wide salivary canal (shown to scale); the labrum containing the food canal (yellow) is scaled down 10-fold for clarity.

Image credit: Frischknecht F, Baldacci P, Martin B, et al. Imaging movement of malaria parasites during transmission by *Anopheles* mosquitoes. Cell Microbiol. 2004;6(7):687-694.

After they are injected into the dermis, sporozoites must navigate to a blood vessel and invade it, an active process that appears to be randomly directed [23]. There are several lines of evidence demonstrating that sporozoites are inoculated into the skin rather than directly into the blood vessel: intravital imaging has allowed for the tracking of sporozoites as they are injected into the skin, move in the skin, and find a blood

vessel [24, 11]; the ability of *Plasmodium* to be transmitted by probing alone [25], and observed delay in sporozoites leaving the bite site [26, 27] are in agreement with the view that sporozoites are not injected directly into the vasculature.

An essential component of sporozoite biology is gliding motility [28]. This form of locomotion does not result in a change in cell shape, nor does it involve appendages such as cilia or flagella [29]; instead, it is a substrate-dependent form of locomotion that is driven by an actin-myosin motor underneath the parasite plasma membrane. This motor propels the sporozoite forward via transmembrane proteins in the thrombospondin related anonymous protein (TRAP) family, which extend from the motor across the membrane and into the external environment where they bind to substrate [30]. TRAP proteins are secreted at the anterior end of the parasite and are translocated to the posterior end as the sporozoite as is propelled forward [30]. Robust gliding, including the efficient cleavage of TRAP within a transmembrane domain, is required for dermal exit [31].

The process of finding a blood vessel in the dermis is estimated to take between a few minutes and three hours [23, 26]. Once in the bloodstream, sporozoites are arrested in the liver, however the efficiency with which they reach the liver is not well-defined and some sporozoites, even after entry into the blood stream, may not reach it. Finally, those that do successfully arrive in the liver must cross the hepatic sinusoid, invade a hepatocyte, and successfully combat the innate intracellular host cell response against invading pathogens [32].

The many barriers for the sporozoites between their production in the oocyst and the successful initiation of a blood stage infection leave many opportunities for any one sporozoite to fail. Each oocyst contains thousands of sporozoites [33], approximately 15% of which reach the salivary gland [9] for potential inoculation into the host. It has been demonstrated that the median number of sporozoites inoculated by the probing mosquito is eighteen [21], and it is likely that at each step between inoculation by the mosquito and replication in the hepatocyte there is a loss in parasite numbers. Although we know little about the initial stages of malaria infection in the mammalian host, it is likely that this stepwise decrease in parasite numbers persists until hepatic replication. There is a decline in the number of sporozoites that successfully exit the skin [11, 28], and likely another decline in the number that successfully invade a hepatocyte.

Plasmodium transmission by Anopheles mosquitoes

Plasmodium parasites are transmitted by the bite of the female mosquito of genus *Anopheles*. While there are nearly 500 species of *Anopheles* mosquitoes, only about 70 are capable of transmitting human *Plasmodium* species, and 41 are implicated as important vectors of the disease [34]. These species are found throughout the world, excluding regions of extreme latitude (approximately >20°S and >50°N) and the Sahara desert [34] placing nearly the entire global population in regions where permissive vectors are present.

The distribution and transmission of malaria depends in large part on the ecology and behavior of the *Anopheles* vector. Climate, weather, human modification of the environment, and seasonality all impact the distribution and success of vector populations. Likewise, the rates of human exposures are impacted by the biting preferences of mosquitoes, the feeding and resting location (indoor vs outdoor), and the time of day the mosquitoes prefer to feed. These entomological and ecological factors intersect with social and human behavior dynamics to create a highly complex and frequently very local system of malaria transmission.

The malaria transmission models of Ronald Ross and George Macdonald

The quantitative aspects of *Plasmodium* transmission have been studied since the early 1900's [3], shortly after the 1897 discovery that Anopheline mosquitoes are responsible for the transmission of the parasite [35]. This work aimed to understand the relationships between the parasite, the mosquito host, and the human host and incorporated the ecological, social, and biological factors that influence transmission. These dynamics are summarized in a collection of malaria models that have evolved over the decades [36]. In the first malaria transmission model, published in 1908, Ross considered the variables that impact the 'infection rate' of malaria in Mauritius. The factors he considered include the average human population (*p*), the proportion of infected persons (*m*), the proportion of individuals with high enough gametocytemia to infect a mosquito (*i*), the number of permissive vectors (a), the proportion of these vectors that feed on a single person in one month (*b*), the proportion of permissive

vectors that can survive for one week (*s*) (note: at the time, one week was considered the minimum time required from bite of an infected individual until the mosquito could transmit to the next host) and proportion of Anophelines that succeed in biting a human during their average life (*f*). These variables were multiplied together to estimate the number of infections in a given region (*fsbaimp*). Of note, Ross discussed the large degree of uncertainty involved with these factors, establishing the need for further study:

> The factors i, *s*, *f*, *b*, are likely to be fairly constant and may be roughly calculated. Thus *i* denotes the proportion of infected persons capable of infecting Anophelines of the proper kind; and we shall not be far wrong if we take it that, on the average, only one quarter of the malaria patients contain enough parasites ripe for this function… Again, *s* denotes the proportion of Anophelines which can survive one week or more… but at a rough estimate it might be put at 1/3… The factor *f* denotes the proportion of Anophelines which succeed in biting humans during one month – that is, I suppose, during their average life… I roughly estimated that half the infected people remain ill after three months…
> Such calculations as these, which may appear far-fetched to many, are useful, not so much for the numerical estimates yielded by them, but because they give more precision to our ideas and a guide for future investigations. [3]

Indeed, in the past century Ross's original model has been revised and improved upon as a greater understanding of transmission dynamics has developed [37–47]. Starting in 1950, Macdonald published a number of papers that greatly enhanced the models proposed in the decades prior [48–50]. While there have been other significant contributions to the field of modeling malaria transmission dynamics, the central role that Ross and Macdonald had in establishing these models is reflected in their common name as "the Ross-Macdonald models" [36].

Three parameters of the Ross-Macdonald models define an important ecological measure— the entomological inoculation rate (EIR). The EIR is a measure of the exposure to infected mosquitoes and thus, the intensity of malaria transmission [51] and is defined as the number of infected bites per person per day [36] or annually [52]. Three variables are multiplied to calculate the EIR: the variable *m* considers the ratio of mosquitoes to humans (*M/H*); the variable *a* reflects the proportion of mosquitoes that feed on humans each day; and *z* is the fraction mosquitoes that are infectious (*Z/M*, the number of infectious mosquitoes divided by the population density of mosquitoes). Estimates of this parameter are frequent in the literature, and over 100 studies in Africa have aimed to compare the EIR of a particular region to the proportion of people who are infected with *Plasmodium* [53]. The 'gold standard' for estimating human biting rates (bites per person per night/year, or *ma*) is human-landing catches, in which individuals capture mosquitoes that have landed on their bare limbs using an aspirator [52]. Estimates of the fraction of mosquitoes that are infectious (*z*), sometimes called the sporozoite rate, are typically calculated after large collections of mosquitoes are analyzed for the presence of sporozoites in the salivary glands, via polymerase chain reaction (PCR) or enzyme-linked immune assay (ELISA), and vary regionally [51].

Closely related to the EIR parameter is the force of human infection [36]. This incorporates one more variable than does the EIR by adding *b*—the proportion of bites by infectious mosquitoes that infect a human—to this equation. This allows for an estimation of the infected bites received by an individual and the probability that the pathogen will establish an infection in the host. The variable *b* was added to

transmission equations in 1933 by Davey and Gordon [54] when they recognized a glaring discrepancy between the numbers of infective mosquitoes and the number of human infections [36]. The addition of this parameter to estimates of the EIR acknowledges that not all infective bites lead to an infection in the host. Host, parasite, and vector characteristics are all likely to impact whether an infective bite will lead to infection, and then whether infection by the parasite will lead to clinical disease [55]. Unlike the measures of the human biting rate and sporozoite rate, the proportion of bites by infected mosquitoes that infect a human has not been directly studied. There has been one attempt to indirectly estimate this proportion by back-calculating from measures of the parasite rate and the entomological inoculation rate in infants, arriving an estimate of between 1.5 and 2.6% of infective bites leading to infection [56]. In 1956, Macdonald briefly addressed the *b* parameter, without giving an indication of the method by which these values were estimated:

> There is also evidence, which appears conclusive to the writer, that in this area only about 1 in every 100 bites inflicted on infants by sporozoite-infected mosquitos resulted in establishment of infection, and in another area only 1 in 20 did so. [57]

Despite these estimates of 1-5% of infective bites resulting in a blood stage infection, human vaccine trials use the bites of five infected mosquitoes to reliably produce an infection in naïve human volunteers [58]. This suggests that these values are likely underestimates of the true rate of infection after each infected bite. However, some of this discrepancy is likely due to the greater salivary gland sporozoite loads of laboratory colonies when compared to wild mosquitos; field-caught mosquitoes rarely contain more than 10,000 sporozoites in the salivary glands [59, 60], while the mean sporozoite

number in a recent vaccine trial was over 35,000 per mosquito [61]. At the other extreme, a figure in *Parasitology Today* suggested that for every 400 infectious bites, there were 200 patient infections [62]. The literature is otherwise lacking in measured or calculated estimates of *b*.

Maintaining the transmission cycle of *Plasmodium* requires not only the successful deposition of sporozoites into the human host, but also the successful establishment of the parasite in the host for later transmission back to the *Anopheles* vector. The rate at which the parasite can establish an infection in the host after infective bite is critical to understanding the relationship between infected mosquito bites and rates of blood stage infection in humans. This addresses an unmeasured parameter in the equations that estimate malaria transmission dynamics.

The following chapters summarize the first studies aimed at estimating *b* in vivo. Using a mouse model, the proportion of infective bites that produce an infection in the naïve mammalian host is presented, based on the intensity of the infection in the mosquito. These results are then related to published data on the number of sporozoites injected into the skin, to propose an estimate for the number of inoculated sporozoites required to produce a blood stage infection. The effect of the duration of mosquito probing, the location that is exposed to mosquito probing, and the acquisition of a blood meal is also presented.

METHODS

Methods for breeding and infecting mosquitoes, source of mice

Anopheles stephensi mosquitoes were reared in the insectary at the Johns Hopkins Malaria Research Institute as described previously [21]: adult female *An. stephensi* mosquitoes were fed a mouse blood meal and allowed to lay eggs on egg traps; mosquito development progressed until the adults emerged from the pupae, which were then placed in cages. The mosquito rearing procedure took place at 27°C and 80% humidity, and adult mosquitoes were fed a 10% sucrose solution. Mosquitoes were infected with *P. yoelii* as described previously [21]: four to six days after emergence, adult mosquitoes were allowed to feed on female Swiss Webster mice infected with *P. yoelii*. These mice were infected via blood transfer from a mouse injected intravenously with dissected sporozoites or by mosquito bites from a previous *P. yoelii* cycle. All experiments with infected mosquitoes took place between 14 and 16 days after the mosquitoes had taken an infected blood meal.

Female Swiss Webster mice were supplied by Taconic Farms (Derwood, MD) and were housed in the animal facility at the Johns Hopkins Bloomberg School of Public Health. The age of the mice used in experiments ranged from 4 weeks to 9 weeks old. Within each experiment, the same age and batch of mice was used. This work was done in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals and the National Institutes of Health. The protocol was approved by the Johns Hopkins University Animal Care and Use Committee (protocols #M011H467 and

#M014H363) which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Single mosquito feed experiments: general methods

Ten days after receiving an infected blood meal, midgut dissections were performed on ≥20 mosquitoes, and oocyst numbers were estimated by phase-contrast microscopy [63]. The proportion of mosquitoes infected within a cage was defined by having at least one oocyst on the midgut. In addition, the number of oocysts present was estimated, and this loosely correlated with the intensity of sporozoite infection of the salivary gland loads four days later. Experiments were conducted using cages that had at least 70% of the mosquitoes infected.

The methods for performing single mosquito feeds is based off similar experiments designed by Medica and Sinnis [21]: fourteen to sixteen days after the infected blood meal, mosquitoes were anesthetized at 4°C and sorted into individual plastic tubes approximately 1 cm in diameter which contained mesh netting at one end. After securing the open end with Parafilm, the mosquitoes were returned to the incubator and were deprived of sugar overnight.

Mice were lightly anesthetized by intraperitoneal injection of ketamine-HCl (35-100 µg/g body weight) and xylazine (6-15 µg/g body weight) and placed on a warming block maintained at 37°C to prevent a drop in body temperature due to anesthesia. A single feeder containing a starved mosquito was placed on the mouse in such a way that the mosquito could bite the mouse through the mesh netting. The mosquito was placed

on the ear and allowed to probe until a blood meal was acquired or she lost interest in feeding. The surface area of the mesh netting was approximately the same size as the ear of the mouse, and was positioned so that it was completely covering the inside of the ear without providing an opportunity for the mosquito to probe on a non-ear surface of the mouse. The feeder was rested against a stationary object to ensure that the mosquito could probe undisturbed. The timing of the probing event was recorded as the cumulative time that the mosquito was on the mouse with the proboscis moving in and out of the skin, ending with the visible start of the blood meal. If the mosquito halted probing, the timer was paused and the mosquito remained under observation until it either began probing again (and the recording of probe time resumed) or the mouse began to wake up as the anesthetic wore off. The acquisition of a blood meal was determined by observing the abdomen of the mosquito for engorgement and red coloration, and confirmed by noting the presence of blood in the esophagus during later dissection. After the completion of the bite, the mosquito was placed on ice and dissected for the quantification of sporozoites. The mice were observed for 15 days and the presence of blood stage infection established by Giemsa-stained thin smear from tail bleeds. Smears were performed on day 5 and confirmed on days 10 and 15; for all mice, the smears on the three days were in agreement.

Variation on the single mosquito feed procedure

Adaptation of single mosquito feeds for probe time studies

Studies investigating the effect of probe time on transmission efficiency were performed in two ways: observational probe time results were collected by monitoring but not controlling the duration of time that the mosquito probed, as described in the previous section. Controlled probe time experiments were conducted by allowing a mosquito to probe on a mouse for exactly ten seconds, one minute, or five minutes, with the same number of mice per probe time duration group. All controlled probe time experiments were performed on the ear. Timing was begun as soon as the proboscis interacted with the skin, and the feeder was removed after the designated time had elapsed. In some instances (one minute and five minute probes only) the mosquito halted probing prior to the end of the designated probe time; these mosquitoes remained under observation until probing resumed or, if the complete probing duration was not met, both mosquito and mouse were removed from the analysis. Occasionally, mosquitoes halted probing and began acquiring a blood meal before the designated probe time had elapsed (five minute probe condition only); in these instances the feeder was gently lifted to disengage the proboscis and the replaced on the ear, forcing the mosquito to resume probing. The timer was paused from the start of blood meal acquisition to restart of probing to ensure that the cumulative probe time was of the required duration.

Adaptation of single mosquito feeds for location studies

For studies investigating the effect of bite location on transmission efficiency, the procedure described in the previous section was performed with the following changes: within each experiment, equal numbers of mice were subjected to the probing of an infected mosquito either to the ear, the tail, or the abdomen. For bites to the tail, the feeder was placed approximately half way down the length of the tail over one of the lateral veins. The surface area on which the mosquitoes could bite was approximately 1/10 cm^2 due to the narrow width of the tail. For bites to the abdomen, the feeder was centered on the ventral surface of the abdomen. In each experiment, the same number of mice were subjected to a bite to the ear, tail, or abdomen.

Methods for quantification of sporozoites in salivary glands

Dissection of salivary glands

Following the experimental procedures described in previous sections, the mosquitoes were dissected and the salivary glands collected as previously described [21]: each mosquito was individually anesthetized on ice, placed in 70% ethanol for 30 seconds, and then rinsed in Dulbecco's Modified Eagle Medium, High Glucose (DMEM; Life Technologies, Grand Island, NY). Using a light microscope and syringe with 25G needle attached, the head was removed and the thorax cut approximately one quarter of the way down to ensure that the all lobes of the salivary glands were collected. Independent experiments showed that the addition of excess mosquito material did not

affect the quantification of the sporozoites (data not shown). This mosquito material was placed in 200 µL DMEM and frozen at -80°C until genomic DNA (gDNA) extraction.

Extraction of genomic DNA from salivary glands

Salivary glands were processed using Qiagen DNeasy Kit in accordance with the manufacturer's recommendations, as described previously [26]. Samples were thawed and lysed via the addition of 12 mAU of proteinase K and proprietary buffer AL, and incubated at 55°C for 30 minutes. The manufacturer recommends an incubation of 10 minutes for nucleated cells, however the incubation time was increased to allow for breakdown of excess mosquito material. After the addition of 200 µL molecular grade ethanol to produce optimal DNA binding conditions, each sample was pipetted onto a silica-based membrane in a spin column, onto which the DNA selectively binds. Two washes were performed to remove contaminants, and the DNA was eluted into water. The elution volume was reduced from the 200 µL recommended by the manufacturer to two elutions of 20 µL in order to increase the DNA concentration and maximize yield. Genomic DNA samples were stored at -80°C until quantification by quantitative polymerase chain reaction.

Development of a standard curve

A standard curve was made from sporozoites which was used to measure the sporozoite loads in the salivary glands isolated from the mosquitoes used for the study. The method is similar to what has been previously reported [21]: sporozoites were

isolated from the remainder of mosquitoes in cages used for experimental feeds (day 14-16 post infected blood meal) by anesthetizing the mosquitoes on ice, placing mosquitoes in 70% ethanol, and rinsing the mosquitoes in DMEM as described previously. The salivary glands were collected by gently disengaging the head from the thorax to withdraw the salivary glands from the body cavity. The salivary glands were next cut cleanly from the head to allow the glands to be removed with minimal mosquito material. This procedure varies from the method used to isolate the experimental salivary glands, for which collecting the entire gland was imperative and excess mosquito material was did not pose a problem. These salivary glands were collected in large quantity (approximately 50-100 per experiment) and placed in a small volume (<100 µL) of DMEM and kept on ice. Using a Squisher™ manual homogenizer, the salivary glands were mushed to break up the clumps and release the sporozoites. The homogenate was centrifuged at 100g for four minutes to remove the large mosquito material, and the sporozoites counted using a haemocytometer. A serial dilution of the sporozoites was performed to yield to following number of sporozoites in 200 µL of DMEM: 50,000; 5,000; 500; and 50, to cover the typical range of salivary gland loads. These 200 µL aliquots with known number of sporozoites were frozen at -80°C until they were processed alongside the experimental salivary glands, as described in the previous section.

Quantification of sporozoites by polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed on the gDNA isolated from the experimental mosquito salivary glands alongside the gDNA derived from the sporozoite standard curve. To ensure consistency across experiments, two standard curves made from different lots of sporozoite dissections were used with each qPCR plate. The primer design and cycling profile has been validated previously [26]. The qPCR was performed in triplicate for each sample, standard, and negative control on the StepOnePlus™ system (Applied Biosystems, Carlsbad, CA) using 12.5 µL SYBR® Green PCR Mastermix (Life Technologies, Grand Island, NY) and 800 nM of primers specific for *P. yoelii* (18S ribosomal RNA; forward primer, 5'-GGGGATTGGTTTTGACGTTTTTGCG-3' and reverse primer, 5'AAGCATTAAATAAAGCGAATACATCCTTAT-3') and 4 µL gDNA in a total volume of 25 µL/well. The cycling profile was 95°C for 15 min followed by 40 cycles of: 95°C, 20s; 60°C, 60s. After amplification, the melting temperature was determined using a dissociation curve to ensure that a single, specific product was formed. The profile for the melt curve was: 95°C for 15s, 60°C for 60s, and incremental increases of 3°C up to 95°C.

Statistical analyses

To determine whether significant differences in rates of blood stage infection were seen between groups of mice exposed to bites by mosquitoes with different salivary gland loads, two-tailed homoscedastic t-tests were used with an alpha-cutoff of 0.05.

To determine whether there was a significant effect of probe time on the probability of infection in observed probe time studies, the number of actual infections was compared to the number of expected infections if there was no effect of probe time, and a chi-squared test used to determine significance. This test was performed on each possible pair of probe times (i.e. <1 min vs 1-3 min; <1 min vs 3-5 min, and so on). To determine whether there was a significant effect of probe time on the probability of infection in controlled probe time studies, Friedman's non-parametric analysis was used; this method ranks the rates of infection across the three probe times for each of the six experiments and calculates a test statistic which is compared to a chi-squared distribution. This method allowed us to compare the probe times across the experiments without the variability between experiments obscuring the differences between groups. This test was performed for both low and high numbers of salivary gland sporozoites (<10,000 sporozoites and ≥10,000 sporozoites per mosquito), as well as all salivary gland loads combined.

To determine whether there was a significant effect of probe location on the probability of infection, the number of actual infections was compared to the number of expected infections if there was no effect of location, and a chi-squared test used to determine significance. This test was performed on each possible pair of locations (ear vs tail; ear vs abdomen; tail vs abdomen) and for both low and high numbers of salivary gland sporozoites (<10,000 sporozoites and ≥10,000 sporozoites per mosquito), as well as all salivary gland loads combined.

To determine whether there was a significant effect of blood meal acquisition on the probability of infection, the number of actual infections was compared to the number of expected infections if there was no effect of blood meal acquisition, and a chi-squared test used to determine significance. This test was performed for both low and high numbers of salivary gland sporozoites (<10,000 sporozoites and ≥10,000 sporozoites per mosquito), as well as all salivary gland loads combined.

RESULTS

Transmission efficiency of single infected mosquito bite

The first specific aim of this project was to establish the proportion of infective bites that proceed all the way to the establishment of a blood stage infection, as an indicator of successful transmission. For each mosquito in this study, we also determined the number of sporozoites in its salivary gland, to determine whether this had an impact on the likelihood of infection.

A single starved *An. stephensi* mosquito infected with *P. yoelii* was allowed to probe on the ear of a Swiss Webster mouse until it acquired a blood meal or lost interest in probing. Following the feed, the salivary glands of the mosquito were dissected and salivary gland load of parasites was determined by qPCR and the mouse was followed for 15 days by Giemsa-stained blood smear. An infected bite was defined as a bite by a mosquito that was later confirmed to contain sporozoites in the salivary glands.

Data from 100 single mosquito feeds is shown in Figure 3. Mice are binned according to the salivary gland load of the mosquito that probed on it. While no clear linear relationship between salivary gland load and the likelihood of mouse infection is evident, there is a higher frequency of infection after bite by mosquitoes that have high salivary gland loads (grades 5+ and 6+, ≥ 10,000 sporozoites per mosquito). To look at this potential difference, we batched the data into groups probed upon by mosquitoes with < 10,000 or with ≥ 10,000 sporozoites in their salivary glands. As shown in Figure 4, the ability of mosquitoes with less than 10,000 sporozoites in their glands to cause a

malaria infection is significantly lower than that of mosquitoes with over 10,000 sporozoites in their glands ($p<0.01$).

Speculations on the proportion of infective bites by infected mosquitoes that infect humans have produced estimates that range from 1-50%; here we provide the first empirical determination of this value measured in a rodent model. We have found that 21% of infected bites result in a blood stage infection in a naïve host, and a significant difference in the proportion of bites causing infection is observed between mosquitoes with < 10,000 sporozoites (12%) and ≥ 10,000 sporozoites (33%) in the salivary glands. While the EIR measures the number of infected bites per person per unit time, thus estimating the *risk* encountered by individuals at various levels of malaria transmission intensity, the estimation provided here of the proportion of these bites that result in a blood stage infection allows for calculation of the 'force of human infections', or the number of infections actually acquired by an individual per unit time.

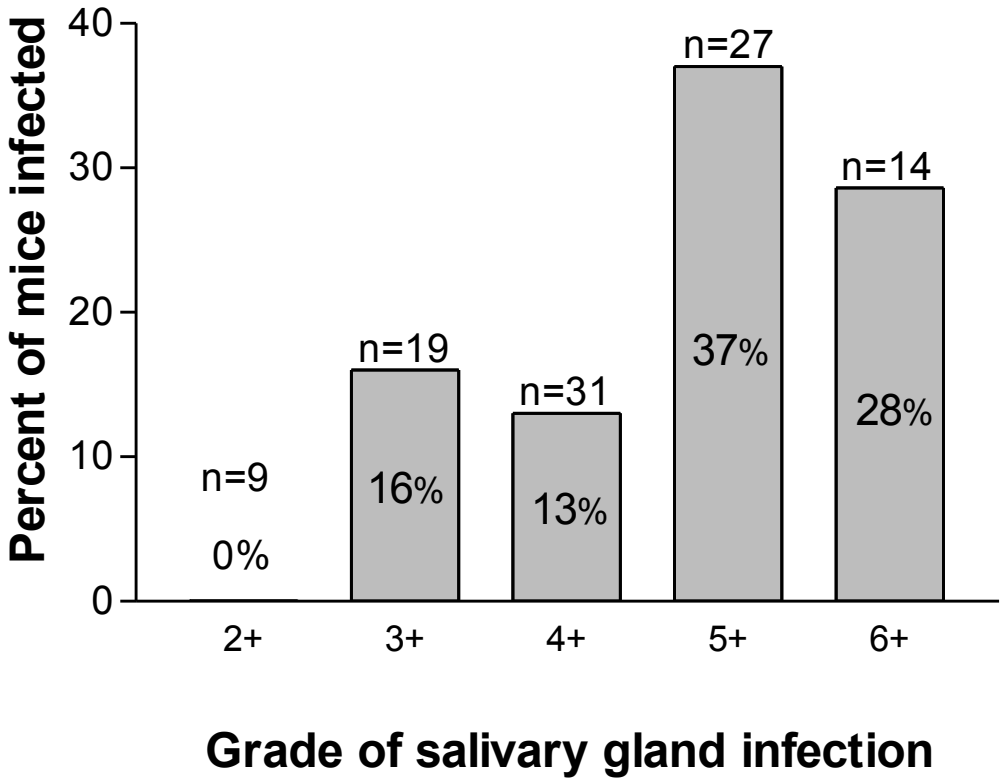

Figure 3. The percent of mice infected with *P. yoelii* after bite by a single infected *An. stephensi* mosquito, batched by the grade of salivary gland infection. The traditional method of grading salivary gland infections is on a log-scale, in which 1+ = 1-10 sporozoites; 2+ = 11-100 sporozoites; 3+ = 101-1,000 sporozoites; and 4+ = 1,001-10,000 sporozoites [64]. This scale has been extended to include 5+ = 10,001-100,000 sporozoites and 6+ = 1,000,000+ sporozoites. The overall percent of mice infected is 21% (n=100).

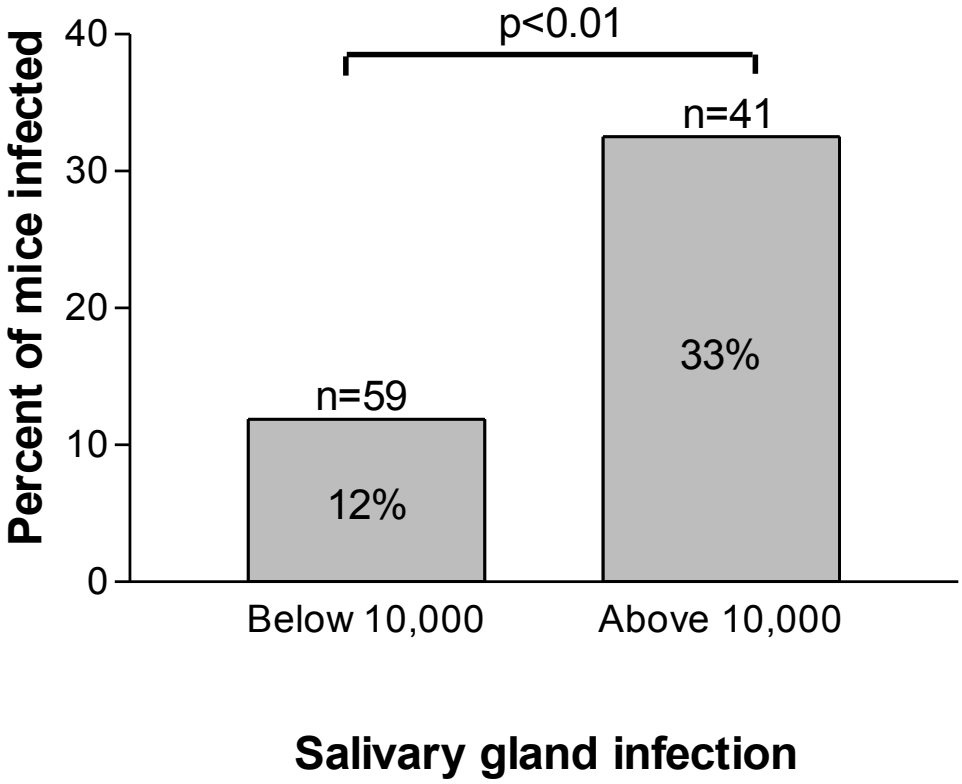

Figure 4. The percent of mice infected with *P. yoelii* after bite by a single infected *An. stephensi* mosquito, batched by mosquito salivary gland load (+/- 10,000 sporozoites per mosquito). When exposed to a bite from a mosquito with over 10,000 sporozoites in its salivary glands, mice are significantly more likely to develop a blood stage infection (p=0.0071).

Transmission efficiency and probing duration

Sporozoites are transmitted to the mammalian host during mosquito probing [25], and it has been suggested that an increase in the duration for which the mosquito probes increases the transmission potential of the parasite [65]. Rossignol et al. discovered that infected mosquitoes probed for a longer duration than uninfected mosquitoes due to sporozoite-induced salivary pathology, which inhibited the ability of the mosquito to locate a blood vessel [65]. This led them to speculate that the pathology caused by the sporozoite may contribute to the efficacy of parasite transmission [65]. While sporozoites in the salivary duct are likely the first to be inoculated during probing, it is not clear whether sporozoites in the acinar cells immediately move into the ducts to replenish those that have left. If they do, then one would expect that increased probing time would result in a greater risk of transmission and subsequent infection. In Figure 5 we show the correlation between probe time and risk of blood stage infection in the original set of 100 single-mosquito feeds in which the probe time was quantified but not controlled. In this dataset, the average probe time was 6.7 minutes and 50% of the observations fell between 3.0 and 8.2 minutes. At the extremes, the shortest duration of probing that resulted in a blood stage infection was ten seconds, and multiple mosquitoes probed for longer than twenty minutes without causing an infection. Overall, there was no correlation between probe time and likelihood of blood stage infection in these experiments.

Since the aforementioned studies were not designed to discern the effects of probe time on transmission risk, a set of experiments was conducted in which the probe

time was the experimentally manipulated variable. This ensured comparative groups of the different probe times, allowed for controlled testing of very short probing durations, and removed the possibility that there were confounding factors between mosquitoes that elected to probe for longer durations and those that transmitted more efficiently.

The probe time was controlled by allowing the mosquito to probe for 10 seconds, 1 minute, or 5 minutes and then removing the feeder. We found no significant differences between the duration of probe time and the likelihood of a mouse developing a blood stage infection (Figure 6), although there is a trend with longer probe times more likely to result in an infection.

Since we have previously shown that salivary gland load affects the likelihood of blood stage infection in the mouse, we analyzed the probe data based on the intensity of salivary gland infection (Figure 7). The same trend is observed, however the differences between the likelihood of infection between probe times remains statistically insignificant.

In neither the un-controlled observations of probe time, nor in the experiments for which mosquito probing duration was precisely controlled, did we observe a statistical difference in the risk of infection based on the duration that the mosquito proboscis was in contact with the mouse ear. A trend towards greater infection frequency with longer probe times was observed in the experiments for which probe time was the experimentally manipulated variable however the trend was not significant when we compared data from six experiments, which included 65 mice in each probe time condition.

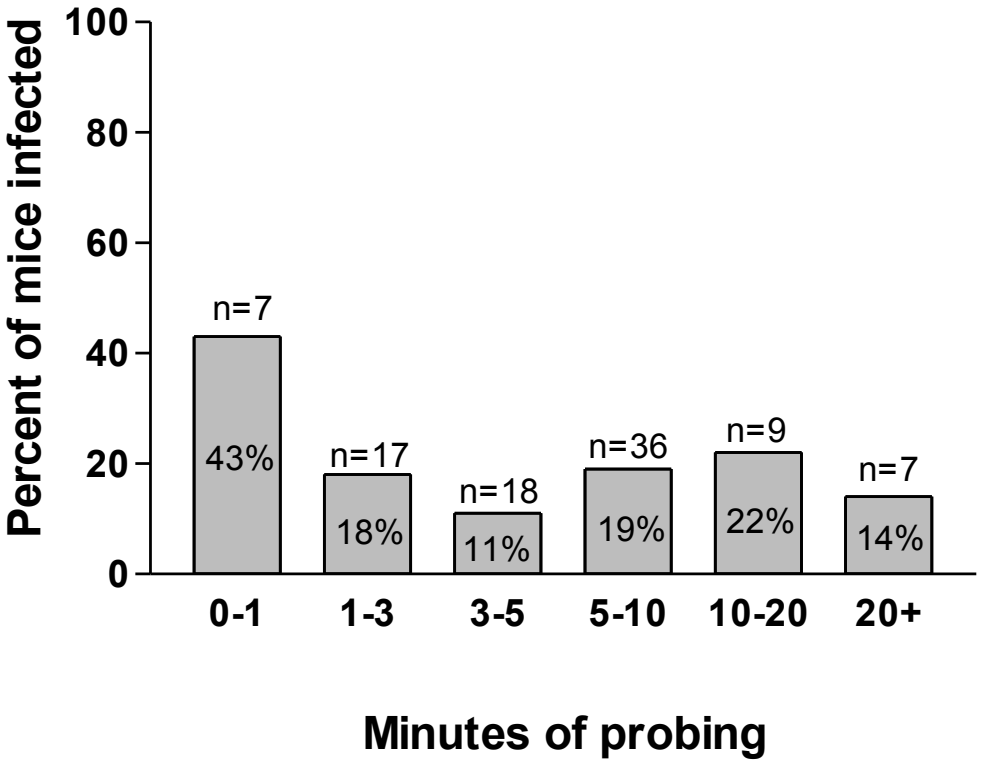

Figure 5. The percent of mice infected with *P. yoelii* after bite by a single *An. stephensi* mosquito, by duration of probing. The mosquito was allowed to probe until it began to imbibe blood or lost interest in feeding. Probe time was not controlled. The percent of mice infected ranged from 11-43%, with no significant differences seen between any groups.

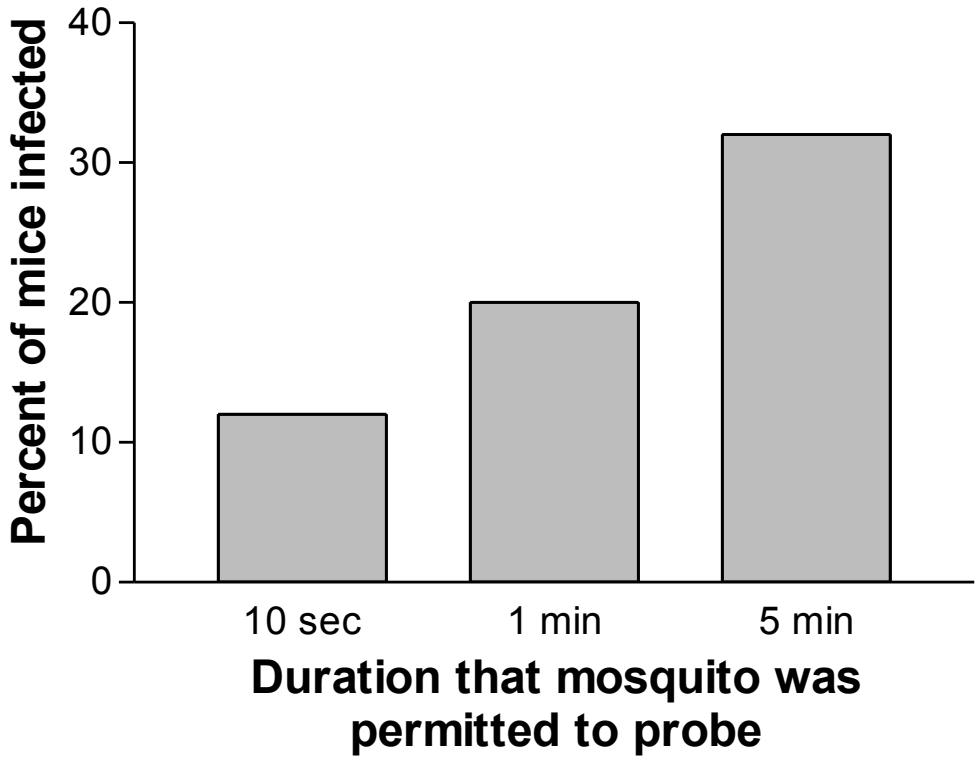

Figure 6. The percent of mice infected with *P. yoelii* after bite by a single *An. stephensi* mosquito that probed for a controlled duration of 10 seconds, 1 minute, or 5 minutes. Bars represent the mean number of mice infected per experiment (6 experiments with 10 or 15 mice in each condition per experiment). All intensities of salivary gland infection are included. Friedman's non-parametric analysis was used to compare the probe times across the six experiments; this resulted in a p-value of 0.0696, which approaches but does not reach statistical significance.

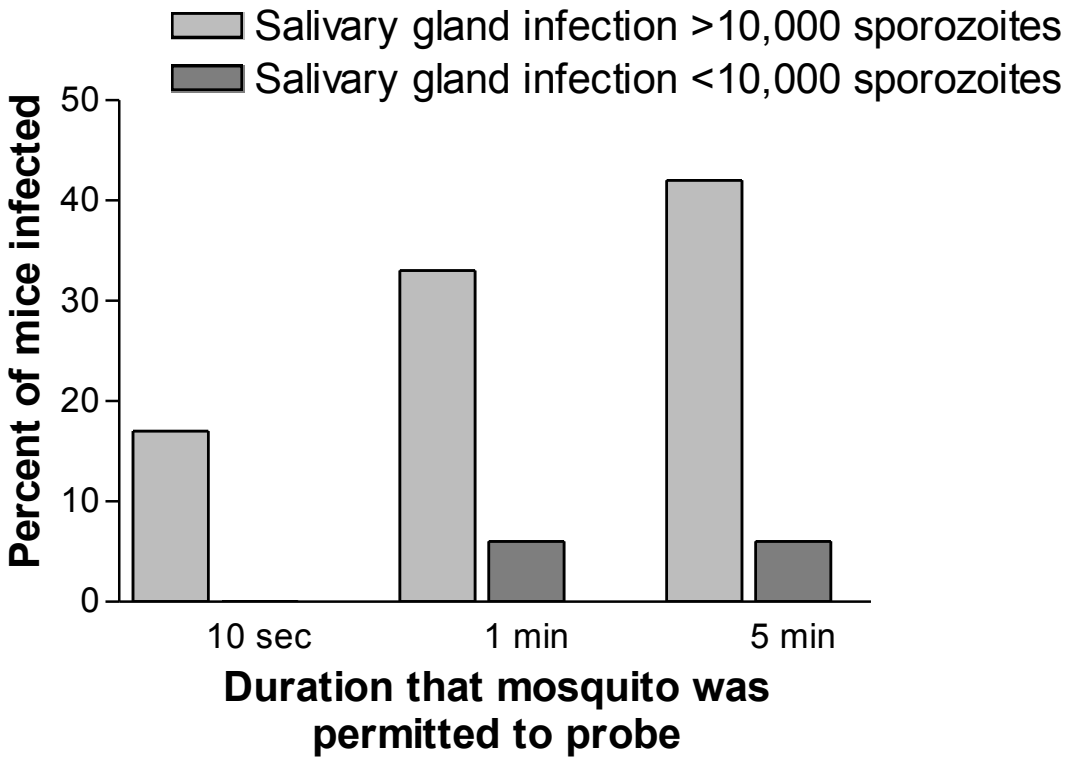

Figure 7. The percent of mice infected with *P. yoelii* after bite by a single *An. stephensi* mosquito that probed for a controlled duration, sorted by intensity of salivary gland load (+/- 10,000 sporozoites per mosquito). Bars represent the mean number of mice infected per experiment. Friedman's non-parametric test for randomized block design was used to compare the probe times across the six experiments, for each salivary gland intensity; the p-values of 0.1148 and 0.0894 for salivary gland infection above and below 10,000 sporozoites, respectively, are non-significant.

Transmission efficiency by region of host exposed to mosquito bite

After their inoculation, sporozoites move in the dermis to locate a blood vessel. The dermal environment encountered by sporozoites can vary dramatically because age, sex and anatomic location can affect the thickness and elasticity of the skin [66, 67]. The thickness of the epidermis on a mouse ear is approximately 12 μm, which is similar to that of the abdomen [68] but less than half the approximate thickness of the tail epidermis (estimates range from 38 μm to 80 μm [68,69]). The human epidermis is somewhat thicker, and ranges from 50-100 μm for thin skin and up to 400 μm in regions such as the palms of the hands and soles of the feet [69]. In comparison, the length of the proboscis is approximately 2 mm in length [70], thus the differing epidermal thickness may not be a factor in sporozoite inoculation and exit. The thickness and vascularization of the dermis are likely to have a more substantial role in the ability of the sporozoite to locate a blood vessel however they have not been well described for various regions of the mouse skin. Here we investigate the role that such variations across skin surfaces may have on the success of sporozoites in escaping the skin.

The thickness and vascularization of the dermis may affect sporozoite success, as it has been shown that sporozoite movement differs in both speed and locomotion type between the ear and the tail of a mouse [71]. As a result, the volume of skin sampled by the migrating sporozoite as it searches for a blood vessel may differ among anatomical locations. To assess the impact that these factors have on the probability of infection, we performed single mosquito feeds on two anatomical locations in addition to the ear.

The data in Figure 8 compares the proportion of infective mosquito bites that resulted in blood stage infection when the mosquito was allowed to probe on different locations on the mouse. For each location, the results are binned according to whether the mosquito had < 10,000 or ≥ 10,000 sporozoites in her salivary glands. The results are compiled from four different experiments and for each experiment, the mice were the same age, and were exposed to the same batch of mosquitoes. In each experiment, equal numbers of mice were used for each bite location.

Despite the differing epidermal thickness and the indication that sporozoite movement may differ between the ear and tail of the mouse [71], and the rates of infection are not statistically different between locations. Given the discrepancy between human and mouse epidermal thickness [69] and likely differences in dermal thickness and vascularization, this may have important implications for the applicability of the results from mouse models to humans.

In these experiments the mosquitoes were allowed to probe until they began to take a blood meal or lost interest. The cumulative probe time was measured, and the proportion of mosquitoes that probed for >5 min and <5 min was plotted for each anatomic location. As shown in Figure 9, mosquitoes biting the tail spent significantly less time probing than mosquitoes biting either the ear or the abdomen ($p<0.01$). We suggest that the increased blood vessel size and proportionally larger blood volume may be responsible for this increased efficiency in finding a blood meal. This may be due to each individual probe having a greater likelihood of coming into contact with a blood vessel.

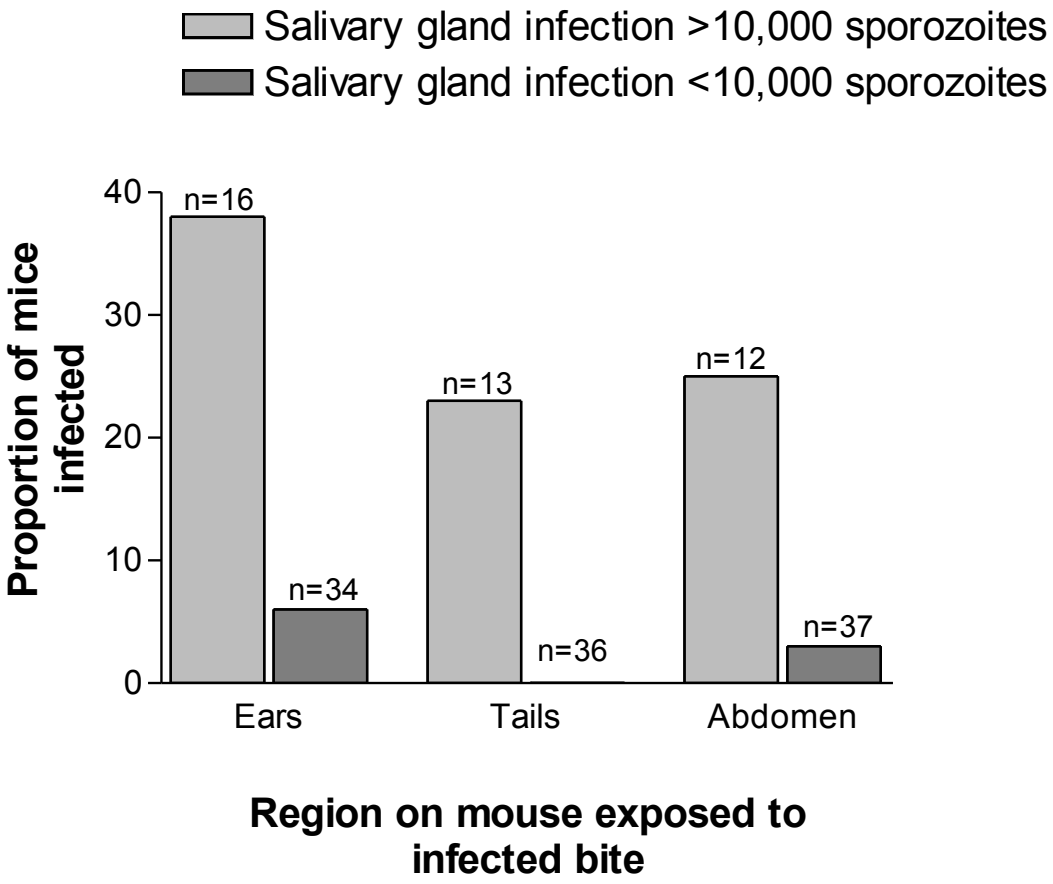

Figure 8. The percent of mice infected with *P. yoelii* after a single bite to the ear, tail, or abdomen by an infected *An. stephensi* mosquito; sorted by intensity of salivary gland load (+/- 10,000 sporozoites per mosquito). The rates of infection are not statistically different between the three locations at either high or low salivary gland infection ($p>0.1$).

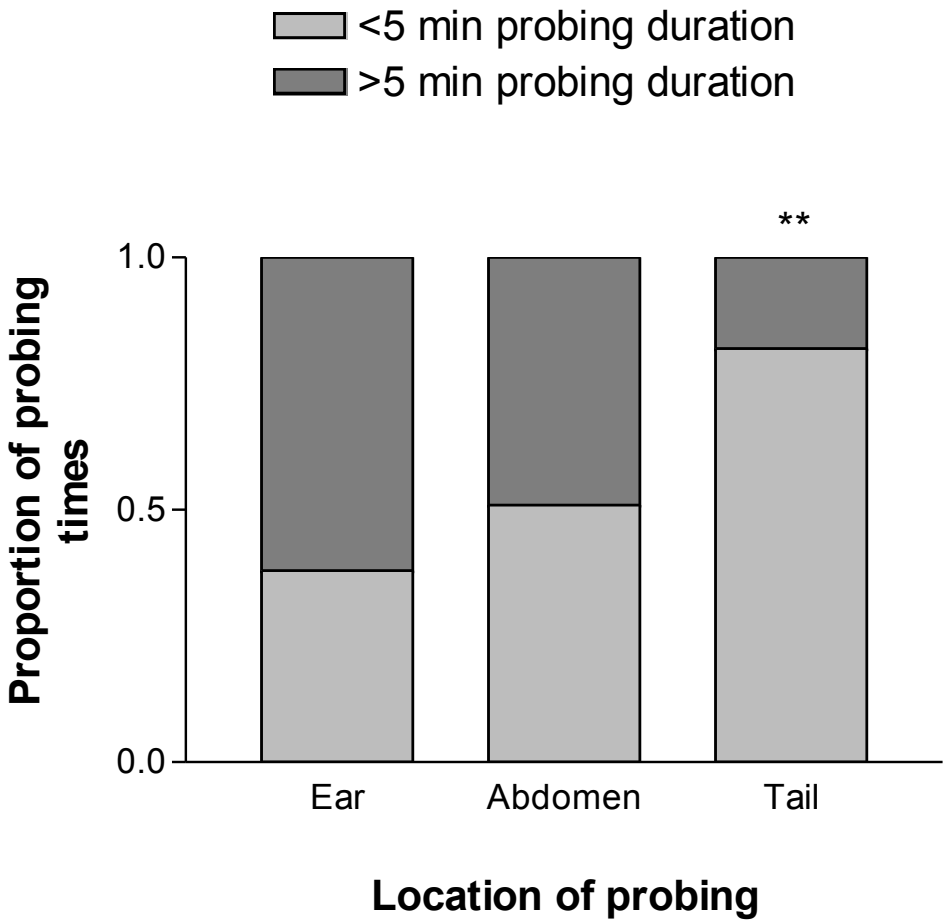

Figure 9. Differences in mosquito probing behavior based on anatomical location. Mosquitoes probed for significantly shorter periods on the tail than on the ear ($p=0.00016$) or abdomen ($p=0.0088$). Time shown is cumulative duration that mosquito proboscis was in contact with the mouse skin, until the mosquito began to imbibe blood or lost interest. $n=39$ for tail and abdomen bites, $n=40$ for ear bites.

Transmission efficiency and blood meal acquisition

Despite increasing evidence indicating that sporozoites are inoculated into the skin and not directly into the vasculature [25, 72], the use of a blood meal as the endpoint for a successful bite in human vaccine studies standard procedure [73, 74, 61]. To further validate the supposition that the transmission of sporozoites is not dependent on the direct contact of the proboscis with the vasculature, we analyzed the rates of blood stage infection in mice exposed to a mosquito that had successfully acquired a blood meal versus mice exposed to a mosquito that had only probed, but not fed, on the mouse. While we cannot confirm that the proboscis did not come into contact with a blood vessel during probing, we expect that, if contact with a blood vessel was required for transmission, the rates of infection would be higher from mosquitoes that had imbibed blood.

Single mosquito feeds were performed as outlined previously with the acquisition of a blood meal recorded. Successful acquisition of a blood meal was defined by visual observation of blood in the midgut of the mosquito, and confirmed during mosquito dissection by examining the esophagus for traces of blood.

The data in Figure 10 shows the rates of blood stage infection in mice that had been probed upon by mosquitoes that successfully found blood versus those that did not find blood. Probe times ranges from 10 seconds to 20+ minutes. The average probe times did not vary significantly ($p=0.18$) between the mosquitoes that had imbibed blood and those that did not (mosquitoes that obtained a blood meal probed for a mean of 5 min 57 seconds, with a standard deviation of 4 min 53 sec; mosquitoes that did not

obtain a blood meal, probed for a mean of 7 min 43 sec with a standard deviation of 7 min 11 sec).

The intensity of salivary gland infection was not similar between mosquitoes that had fed and those that had only probed. For mosquitoes that fed on the mouse, 29% had more than 10,000 sporozoites in the salivary glands; of mosquitoes that probed but did not feed on the mouse, 55% had more than 10,000 sporozoites in the salivary glands. Further, the average gland load for heavily (>10,000) infected glands was 45,000 in mosquitoes that had fed, and 105,000 in mosquitoes that only probed. An interesting possibility based on these data is that mosquitoes with very high salivary gland sporozoite loads may experience more difficulty in obtaining a blood meal. This is in agreement with the findings of Rossignol et al. who determined that mosquitoes experience a sporozoite-induced pathology that results in increased difficulty in finding a blood vessel during probing [65]. Here we expand upon this concept, suggesting that this pathology may be directly proportional to salivary gland load, with higher gland loads causing more difficult in finding a blood vessel than lower gland loads.

Despite the observation that mosquitoes with especially heavy infections are less likely to obtain a blood meal, the percent of mice that became infected, by blood feed status, when categorized by salivary gland infection did not differ (Figure 11). This analysis provides further evidence that there is no difference in mouse infection rates when infection was transmitted by a mosquito that took a blood meal versus one that only probed.

Previous studies have indicated that probing is sufficient for transmission to take place [25] and here we expand on this observation to show that blood meal acquisition is not only noncompulsory, but does not result in any increase in the likelihood of blood stage infection.

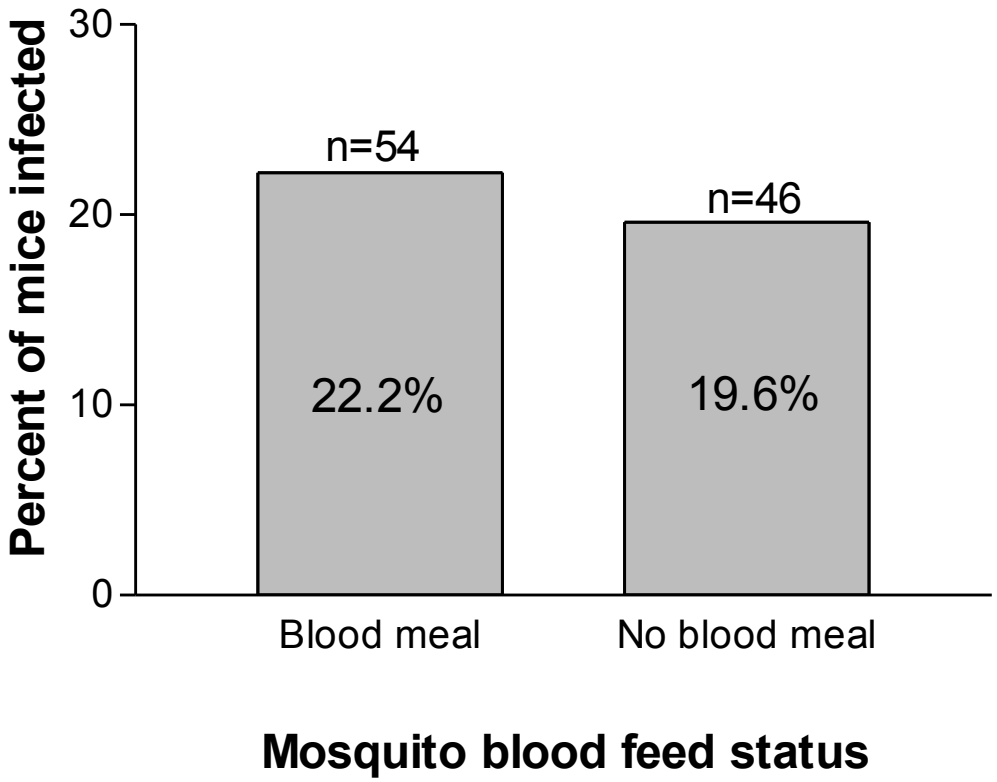

Figure 10. The percent of mice infected with *P. yoelii* after bite by a single *An. stephensi* mosquito by mosquito blood feed status. The proportion of mice that developed a blood stage infection after being probed upon by a single infected mosquito that took a blood meal is not different from the proportion infected when the mosquito did not succeed in obtaining a blood meal (p=0.745).

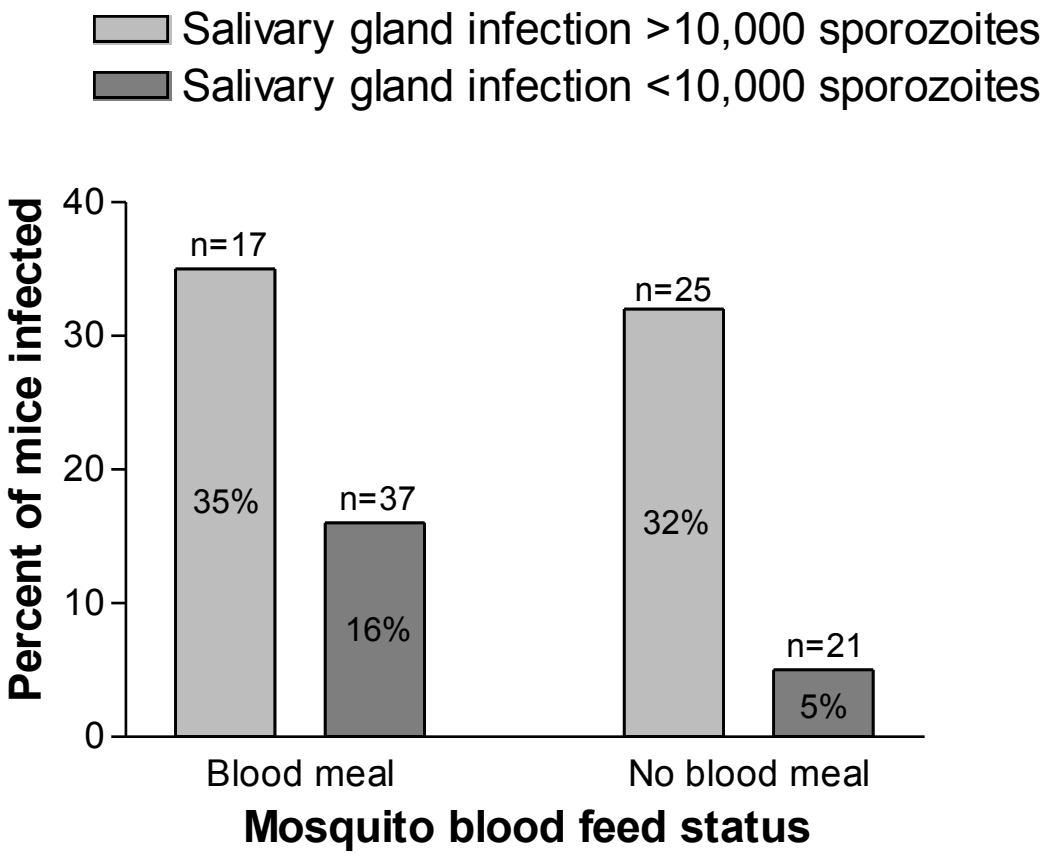

Figure 11. A comparison of mice that developed a blood stage infection after bite by infected mosquitoes by mosquito blood feed status, and by intensity of salivary gland infection (+/- 10,000 sporozoites per mosquito). For each above and below 10,000 sporozoites per mosquito, no significant difference is seen between the proportion of mice infected by mosquitoes that bad taken a blood meal or only probed ($p>0.2$)

Overall transmission efficiency of infected bites to the ear

Our initial study of 100 mice exposed to a single infected bite to the ear was followed by an investigation of the effects of probe time on transmission efficiency (186 ear bites) and a comparison of three bite locations (40 ear bites). The percent of the 186 mice infected after exposure to a single infected mosquito bite of controlled probing duration was 22%, providing a robust validation of the original value (21%) determined by the initial 100 mice in which the probe time of mosquitoes was not manipulated. The study of 40 mice exposed to a bite on the ear against which tail and abdomen bites were compared had a somewhat lower percent infection of 10% (this was due, in large part, to a high proportion of mosquitoes with low salivary gland loads in this study). When all sources of single infected bites to the ear are pooled and all salivary gland loads included, the overall rate of infection in 326 mice is 20%. Figure 12 presents this compilation of all 326 bites, by intensity of salivary gland infection.

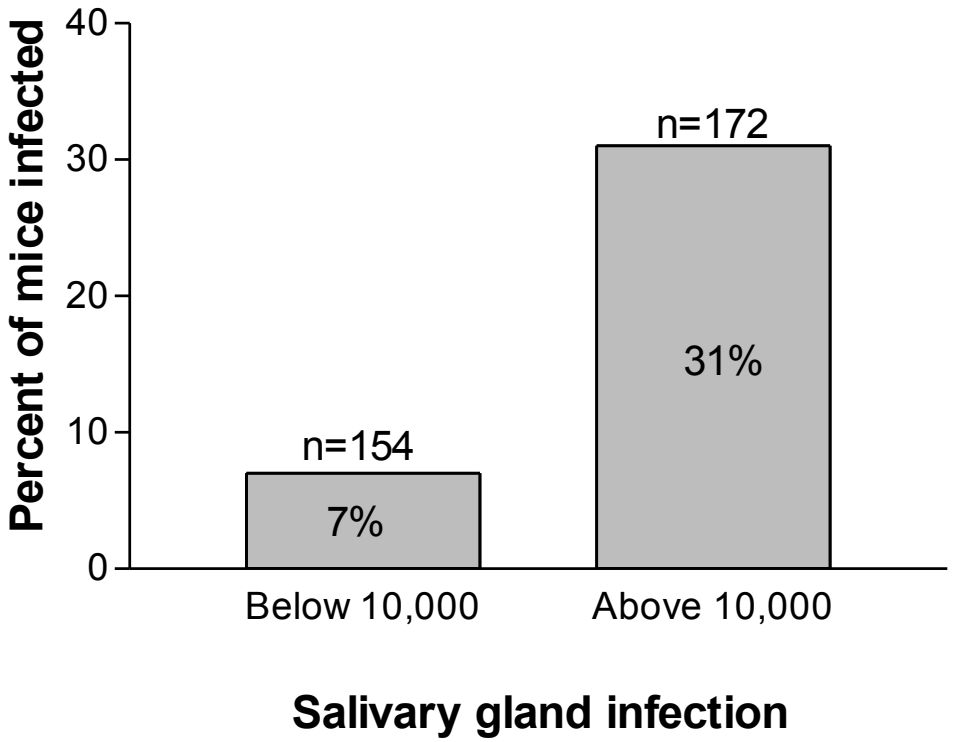

Figure 12. The percent of mice infected with *P. yoelii* after bite by a single infected *An. stephensi* mosquito, batched by whether salivary glands have a heavy or light/moderate infection (+/- 10,000 sporozoites per mosquito) for all bites to the ear (n=326). Overall percent of mice infected is 20%, and includes 140 mice exposed to a mosquito that was permitted to probe until it lost interest or began to imbibe blood and 186 mice exposed to mosquitoes that were permitted to probe for predesignated durations.

DISCUSSION

The efficiency of *Plasmodium* transmission, from infected salivary gland to blood stage infection, has been described here for the first time using a laboratory model. We estimate that the proportion of infective bites that result in at least one sporozoite making it past all potential barriers to infection of the host— inoculation into the skin, escape from the skin into the vasculature, arrest in the liver, invasion of a hepatocyte, development in the hepatocyte and final exit to establish a blood stage infection—is 21%, when bites from mosquitoes with heavy and low salivary gland loads are pooled. While the EIR measures the number of infective bites per person per unit time, thus estimating the *risk* encountered by individuals at various levels of malaria transmission intensity, the estimation of the proportion of these bites that result in a blood stage infection allows for measurement of the 'force of human infections', or the number of infections actually acquired by an individual per unit time. Speculations on the proportion of infected bites that lead to human infection have produced estimates that range from 1-50% [56, 62]. Here we provide the first empirical determination of this value measured in a rodent model.

Our value of 21% of infective bites resulting in a blood-stage infection is not surprising, given that malaria vaccine trials require 5 infectious mosquitoes to reliably produce an infection in naïve volunteers. While there has been one suggestion that fewer mosquitoes are sufficient to produce an infection when using aseptically reared mosquitoes—with the bites of three infected mosquitoes causing infection in 100% of volunteers and the bite of only one infected mosquito causing infection in 83% of

volunteers [58]—only mosquitoes that had successfully acquired a blood meal from the host were considered. Any mosquitoes that had merely probed without ingesting blood were replaced, despite increasing evidence that probing is sufficient to transmit sporozoites. This resulted in a mean exposure of 6.7 mosquitoes per participant in the group reported to have been bitten by three mosquitoes, with all participants exposed to a minimum of five mosquitoes [58].

In light of our observation that there is no relationship between blood meal acquisition and risk of infection, the use of blood meal ingestion as the endpoint for mosquito bites in human vaccine trials could skew trial results. In such studies, malaria challenge consists of exposure to five infective mosquito bites, and the mosquitoes are then dissected to confirm salivary gland loads of at least 1,000 sporozoites and the presence of blood in the abdomen [73]. Exposing all volunteers to the same number of infective mosquitoes is presumably an attempt to expose all participants to a similar dose of challenge sporozoites, but the current protocol requires that each mosquito have ingested blood to be counted among the five bites constituting an exposure. This criterion results in volunteers being exposed to varying numbers of infective mosquitoes before the requisite five have successfully fed. A recent trial has had a range of 9-27 mosquitoes placed on an each individual volunteer before five have consumed blood [58], resulting in what may be a three-fold range of actual sporozoite exposure. Based on our results demonstrating that blood meal acquisition is not required for transmission to take place, and that there is *no* increase in transmission efficiency with the acquisition of a blood meal by the mosquito, we propose that the challenges

experienced by vaccine trial volunteers are as varied as the number of mosquitoes that have probed. The observation that the acquisition of a blood meal does not increase the likelihood of infection is further supported by our reports of infection rates after exceptionally short probe times. For the 186 mice that had a restricted probing duration of 10 seconds, 1 minute, or 5 minutes, no mosquitoes were permitted to feed, and yet the overall rate of infection did not differ from conditions in which mice were permitted to imbibe blood. As such, a new model—one in which mosquito probing and not feeding is used as a marker of exposure—is needed to standardize challenge dose of sporozoites among vaccine trial volunteers.

It has been observed that mosquitoes that are infected with *Plasmodium* sporozoites probe for a longer duration when searching for blood [65], leading some to propose that the parasite manipulates the behavior of the mosquito vector to increase its fitness, by lengthening the probe time of the infected feeding mosquito and thereby increasing the likelihood of sporozoite transmission [65, 75–77]. We have shown that an increase in probe time only slightly increases the likelihood of infection if at all (the trend was not statistically significant). These studies add an important component to this growing understanding of the temporal dynamics of sporozoite transmission, since none of the previous studies have used the development of blood stage infection—the endpoint of sporozoite inoculation—as the readout for successful transmission.

These results may also have implications for the movement of sporozoites in the mosquito immediately prior to transmission. Imaging sporozoites in the acinar cells of the mosquito salivary glands is not yet possible, and the behavior of the parasite as it

traverses the ducts from the distal salivary glands to the main salivary duct is not well understood. While we did not assess the movement of sporozoites through these regions directly, our observation that there is only a slight increase in infection risk over a five minute probing period suggests that the regeneration of sporozoites is not continuous over this period of time. This supports previous work suggesting that salivation [65] and sporozoite inoculation [18, 22, 78] are not greatly impacted by the duration of probing. However, the increased likelihood of transmission from salivary glands with ≥ 10,000 sporozoites, combined with the trend of increased likelihood of transmission with longer probe times, suggests that some regeneration may be occurring. The incremental increase in infection likelihood over 10 seconds, 1 minute, and 5 minutes, although not significant, suggests that the salivary duct sporozoites may be regenerated in a slow but linear fashion. Quantification of the inoculated sporozoites after these durations of probing is an area for further research.

The need for five infected mosquitoes to reliably produce an infection in naïve vaccine trial volunteers has predicted that not all infective bites will result in an infection and this assumption has been demonstrated by our laboratory studies. However, just where along the pathway from infected bite to blood stage infection approximately 80% of infective bites “fail” is not clear. It has been demonstrated that approximately 20% of infective bites do not result in the inoculation of sporozoites [18, 21, 78], accounting for some of this failure to produce an infection. Three other locations may present a challenge to the sporozoite: exiting the dermis into the blood circulation [11], arrest in the liver and crossing of the sinusoidal barrier, successful

invasion and development in the hepatocyte [32, 12, 79] and the escape of merozoites from the hepatocyte [80]. The efficiency of the sporozoites at each pre-erythrocytic step after inoculation is of great importance to better understanding malaria transmission dynamics, and is summarized in Figure 13.

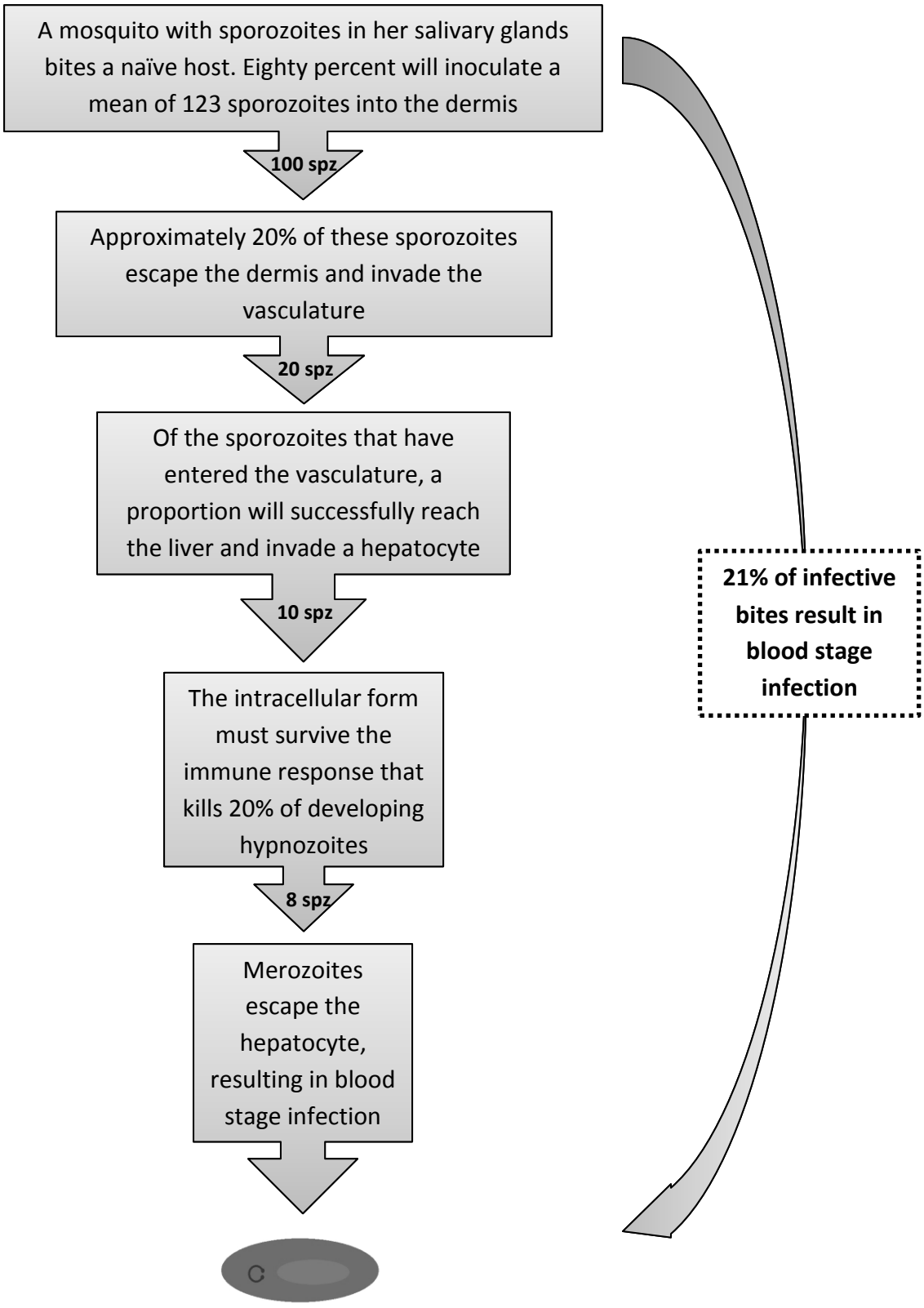

Figure 13. A schematic of the efficiency of sporozoites at each potential barrier to host infection

Figure 13: A schematic of the efficiency of sporozoites at each potential barrier to host infection. For every 100 infected bites, approximated 80 will inoculate sporozoites into the dermis of the host [18, 21, 78], however we can found that only 21 will result in blood stage infection. In bites that inoculate sporozoites, a mean of approximately 100 are inoculated [21]. It has been suggested that of inoculated sporozoites, 20% escape the skin [81], and half of these sporozoites reach and invade a hepatocyte, and another 20% are killed during hypnozoite development (P. Sinnis, personal communication, 2015).

When our observation that 21% of bites lead to infection is combined with the current understanding of these barriers for the sporozoite throughout its journey from inoculation to escape from the liver, we estimate that a minimum of 100 sporozoites must be injected into the skin to produce a blood stage infection.

Our study has also made a crucial observation regarding the relationship between salivary gland load and transmission potential. There is some dispute this relationship, and studies measuring the inoculum of probing mosquitoes have estimated that 75-80% of infectious bites will result in the inoculation of sporozoites, with no consistent correlation to salivary gland load [18, 21, 78]. We found only a weak correlation between number of sporozoites in the salivary glands of the mosquito and the risk of blood stage infection in the mouse over a range of salivary gland loads however at especially high gland loads (over 10,000 sporozoites per mosquito) the increased likelihood of infection is highly significant. In the first study to directly measure the number of sporozoites inoculated into the skin of a live mouse by a probing mosquito, Medica and Sinnis reported a poor correlation between the number of *P. yoelii* sporozoites in the salivary glands of *An. Stephensi* mosquitoes and the number of sporozoites inoculated [21]. This supports previous data indicating little to no correlation between gland load and inoculum when mosquitoes are artificially stimulated [18, 82] or urged to probed through detached mouse skin [78]. While a strong consistent correlation was not observed in any of these studies, there is some indication that at especially high salivary gland loads, a greater number of sporozoites are inoculated [18, 21]. When this same cutoff of is applied to the previously published data reporting sporozoite inoculum, a higher average number of sporozoites is found in the ears of mice probed by mosquitoes with ≥ 10,000 salivary gland sporozoites than found in mice exposed to probes with lower salivary gland loads (average of 62

sporozoites for salivary gland loads < 10,000 and average of 144 sporozoites for salivary gland loads ≥ 10,000 sporozoites; reanalysis of Medica and Sinnis, 2005).

Two explanations are offered for the observation that bites from mosquitoes with especially high salivary gland infections are more likely to result in the development of an infection. At all stages of initial infection – escape from the inoculation site, arrest in the liver, invasion and development in the liver – there is likely to be a 10 to 50% decrease in the number of sporozoites that make it past each step. Thus, the large number of sporozoites in the salivary glands may be required to overcome this inefficiency of individual sporozoites. An alternative explanation is that there are characteristics of the sporozoites that successfully invade the salivary glands that make them superior invaders. Thus, a high numbers of sporozoites in the salivary glands may result in a greater likelihood of infection, not because a large number of sporozoites are required for infection, but because the sporozoites that are found in heavily-infected glands are better suited to invade the vasculature and liver. This is an important avenue for future investigation.

While the reasons behind this observation—that transmission by mosquitoes with high salivary gland sporozoites is more efficient than by mosquitoes with low salivary gland loads—are unclear, the relevance of this observation to the levels of salivary gland infections in the field is paramount. Quantitative studies on the numbers of sporozoites in the salivary glands of *Anopheles* vectors in the field are limited, however indicate that only a small segment of field mosquitoes contain more than 10,000 sporozoites [59, 60]. This implies that a relatively small proportion of mosquitoes

are highly efficient vectors; since our data indicate that transmission by mosquitoes with less than 10,000 sporozoites in their salivary glands have a transmission efficiency of only 7 to 12%, it is suggested that most bites by infected mosquitoes do not result in a malaria infection.

To acquire this first estimation of the proportion of infected mosquito bites that result in blood stage *Plasmodium* infection, we used a rodent system involving Swiss Webster mice and the non-lethal mouse parasite *P. yoelii*. For practical and ethical reasons, such studies cannot be performed in humans, and the use of this system allows for an initial estimate for how biting rates may relate to infection rates in humans. While the use of rodents as a surrogate for humans in these studies to understand malaria transmission dynamics may not be a perfect reflection of transmission in humans, there is evidence to suggest that the transmission dynamics of *P. yoelii* and *P. falciparum* are quite similar [61, 83].

As the first study assessing these dynamics, we have aimed to reduce as much variation as possible, simplifying the system to acquire an initial estimate of *b*. Future research that better capitulates the variables that may affect rates of infection in human populations are needed to better understand the applicability of this data to the field. Two such variables are discussed below:

The research describes here used only naïve mice, however in areas of high malaria transmission, individuals may be exposed to as many as 300+ infected bites per year [52]. It will be important to understand how the risk of blood stage infection changes with repeated exposure to infected mosquito bites In addition, it is well known

that only a very small fraction of mosquitoes in a given region are infectious [52]. Both the proportion of infectious mosquitoes (*z* in the Ross-Macdonald equations) and the human biting rate (*ma*) can vary dramatically; because the sporozoite rates vary regionally but are typically low [52], individuals receive many times the number of uninfected mosquito bites as infective bites. There are indications that uninfected bites can prime the immune system [81, 84], which may have a small effect on the ability of sporozoites to escape the dermis. The changing transmission efficiency of *Plasmodium* parasites after an individual is exposed to varying numbers of infected and uninfected mosquito bites is an important area of research that is necessary for understanding how the estimates described here apply to human populations.

This first determination of the proportion of infective bites that result in an infection of the host allows for the calculation of the force of human infections and the basic reproductive rate, among other measures of transmission, which could not be estimated previously. The similar likelihood of transmission success in very short probe times when compared to extensive probing, and in the absence of blood meal acquisition, indicate that transmission risk may be concentrated early in the biting event, and call into question the use of a blood meal as a marker of transmission risk in malaria vaccine studies. We have also demonstrated that the number of sporozoites in the salivary glands of infected mosquitoes has an effect on infection risk after bite, emphasizing the need for quantitative measures of the intensity of the sporozoite rate in field populations. This quantification of the relatively low transmission efficiency of *Plasmodium* sporozoites after an infected bite has implications for malaria control

efforts and provides a long-overdue contribution to the understanding of sporozoite efficiency in malaria transmission dynamics.

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Developed extensive skills in experiment design, collecting and analyzing data, and adjusting methods to meet experimental goals as part of self-directed master's thesis work studying the rates of blood-stage *Plasmodium yeolii* infection a mouse model after exposure to single infected mosquito bite.

Accomplishments include:

- Using a rodent model, determined the risk of blood stage malaria disease after a single infected mosquito bite, allowing for the first calculations of the force of human infections as a measure of risk.
- Established the contributions of salivary gland sporozoite load, probe time, probe location, and blood meal acquisition on infection risk
- Strong presentation skills and novel research contributions are recognized by the selection of this work for presentation at the Johns Hopkins Malaria Research Institute seminar series (February, 2015).

Skills include:

- Isolation and characterization of *Plasmodium* species including performing cycles in mosquitoes and mice, dissection of mosquito mid guts and salivary glands for visualization of oocysts and sporozoites, rapid collection of salivary gland sporozoites for use in live-imaging assays, and microscopy of blood stage and sporozoite forms.

- Proficient in rodent handling including anesthesia and euthanasia, cardiac punctures, and tail-vein/intraperitoneal/subcutaneous injections for experimental procedures and parasite cycling.
- Strong background in mosquito rearing principles: knowledge of materials and environmental conditions optimal for breeding *Anopheles stephensi, A. gambiae,* and *Culex* mosquitoes; identification of mosquitoes by species and sex; and infection with Plasmodium parasites by mouse bite and membrane feeding assay.
- Expertise performing quantitative PCR, traditional PCR, and DNA gel electrophoresis.
- Strong data organization and analysis skills, including proficiency with R programming and analysis, GraphPad Prism, and ArcGIS.
- Excellent presentation and written communication skills, an aptitude for displaying data in clear manner and tailoring results for diverse audiences, and exemplary interpersonal skills with students, colleagues, and superiors; experience in manuscript, poster, abstract, and funding application preparation.

UNIVERSITY OF VERMONT **JANUARY 2011 – AUGUST 2013**

DEPARTMENT OF BIOCHEMISTRY

Laboratory Research Technician

Member of large, diverse, research team investigating the haemostatic properties of blood coagulation.

Accomplishments and expertise:

- Designed, conducted, analyzed, and interpreted primary research comparing two diagnostic instruments, leading to peer-reviewed publication (first author).
- Assessed the procoagulant activity of red blood cells after storage; designed and performed experiments using thromboelastographic instruments (manuscript in review, first author).
- Wrote and edited a Manual of Operations (100+ pages) for the collection and processing blood samples from trauma patients for use in research institutions nationwide as well as by the Department of Defense; optimized protocols for inclusion in manual and conducted evaluations of protocol implementation and data validity.
- Trained approximately 12 technicians and emergency room personnel in research blood collection and processing; organized incoming blood specimens.

- Presented progress updates to Primary Investigators and Department of Defense personnel; prepared written materials to distribute to collaborators; organized the manufacture and transportation of reagents and samples between research sites; managed the Institutional Review Board approval process for multi-center research study.
- Proven skills in biochemical techniques including gel electrophoresis, immunoblotting, column chromatography, protein isolation, fluorogenic assays, and plasma clotting assays
- Extensive experience handling biohazardous and sterile materials including performing phlebotomy and working with whole blood.
- Advanced knowledge of thromboelastographic instrument operation, leading to the training and supervision of research personnel

PUBLICATIONS

Aleshnick, M., Orfeo T., Brummel-Ziedins K., Gissel, M., and Mann, K. (2014) Interchangeability of rotational elastographic instruments and reagents. *J. Trauma and Acute Care Surgery* 76: 107-13.

Aleshnick, M., Foley, J., Keating, F. and Butenas, S. Procoagulant activity in stored units of red cells. *Submitted for publication.*

TEACHING EXPERIENCE

JHSPH DEPTARTMENT OF MICROBIOLOGY AND IMMUNOLOGY **OCTOBER 2014 – DECEMBER 2014**
Teaching Assistant for Biology of Parasitism

- Designed and conducted lab experiments with students, assisted with microscopy labs, prepared written lab materials

PRIVATE TUTOR, BURLINGTON VT AND BALTIMORE, MD **JUNE 2010 – PRESENT**

- Ongoing tutoring of high school, college, and continuing education students in chemistry, biology and mathematics

UNIVERSITY OF VERMONT ACADEMIC SUPPORT PROGRAMS **AUGUST 2008 – MAY 2010**

- Physics and Chemistry Tutor
- Supplemental Instructor – General Chemistry

VOLUNTEER EXPERIENCE

GREATER HOMEWOOD COMMUNITY CORPORATION **SEPT EMBER 2014 – PRESENT**
Adult Literacy Tutor

Worked on-on-one with adult learners in Baltimore, MD preparing students to take the GED exam; engaged in long-term mentoring and support to ensure the educational success of underrepresented minorities.

JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH **SEPTEMBER 2013 – PRESENT**
Dept. of Molecular Microbiology & Immunology and the JH Malaria Research Institute

- Student-faculty liaison
- Student host for external speakers

BIOMEDICAL SCHOLARS ASSOCIATION **JUNE 2014 – PRESENT**
Vice President

- Executive board member of student organization aimed at increasing the academic, professional, and social opportunities of multicultural students and celebrating diversity
- Representative for Johns Hopkins Medical Institutions Equality in Medicine Committee
- Led workshops for high-school students pursuing careers in science and medicine

COMMUNITY ADOLESCENT SEXUALITY EDUCATION **JANUARY 2014 – JUNE 2014**
Volunteer Classroom Teacher

- Collaborated with public school professionals to provide comprehensive sexual health education to 7th grade students, including drug and alcohol safety and violence prevention. Revised curriculum and provided ongoing mentoring to underprivileged adolescent population

FLETCHER ALLEN HEALTH CARE **JUNE 2011 – JUNE 2012**
Volunteer

- Provided support for pediatric inpatients and their families and helped them navigate the Fletcher Allen Health Care system.
- Conducted infection control research throughout hospital system to assess hand-washing compliance among healthcare staff, resulting in revised protocols and increased training for personnel.

COURSE HIGHLIGHTS, CERTIFICATES, AND TECHNICAL QUALIFICATIONS

- Certificate in **Vaccine Science and Policy** (JHSPH, May, 2014)
- Certification in **Good Clinical Practice** (JHSPH, May, 2014)
- Certificate in **Healthcare Epidemiology and Infection Prevention and Control** (JHSPH, December, 2014).
- Certificate in **Pharmacoepidemiology and Drug Safety** (JHSPH, February, 2015)
- Certification in the **Protection of Human Subject in Research** (UVM)
- Training in rodent handling and safe insectary practices (JHSPH)