NEGATIVE EFFECTS OF HOST SEROCONVERSION ON MOSQUITO REPRODUCTIVE POTENTIAL

by

RYAN BROWN

(Under the Direction of Donald Champagne)

ABSTRACT

Seroconversion is well known to occur in the hosts of mosquitoes. In previous studies, the process of seroconversion has been shown to have negative effects on the fitness of other arthropods. My research has shown that seroconversion also negatively affects mosquitoes, reducing the volume of their blood meals, the number of eggs they can produce, and the number of those eggs that are viable. Seroconversion was confirmed by western blot analysis. The effects were seen with all four species used in this study (*Aedes aegypti*, *Aedes albopictus*, *Anopheles stephensi*, and *Anopheles gambiae*). Some level of cross reactivity was also observed as members of the same genus negatively affected each other.

When the experiment was repeated using B-cell knockout mice (mice unable to seroconvert), there was no observed fitness reduction. This result confirms the hypothesis that host seroconversion is necessary for the observed fitness reduction to occur.

INDEX WORDS: Aedes, Anopheles, seroconversion, fitness, mosquito, scapegoat, blood feeding, saliva

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DEDICATION

First off, I need to dedicate this to my mom as she provided millions

Upon millions of dollars of support through out this endeavor.

Consequently, I need to thank all my sisters…all six of them.

Kindly, I also need to thank all my Entomology friends…you know who you are.

More importantly, I need to dedicate this to my committee, whose doors were

Always open when I needed a helping hand.

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Without which this project would not be possible.

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CHAPTER 1

INTRODUCTION

Mosquitoes serve as important vectors for the transmission of several parasitic and viral infections in humans, including malaria, filariasis, encephalitis, yellow fever, dengue, and many others (17, 30, 36, 44, 55). There are approximately three and a half thousand species in the family Culicidae [Diptera] (17, 36). Culicids are one of the more primitive families in the order Diptera, and they can be found on every continent except Antarctica (17, 36). They were the first arthropods formally determined (in 1878) to be intermediate hosts of vertebrate parasites (36), and arguably they are the most important vectors affecting human health since they are the most abundant blood-feeders (30), and they transmit a larger variety of pathogens than any other arthropod, with the possible exception of ixodid ticks. Some species exhibit considerable host specificity, while others are more generalist feeders. Culicids are known to feed on amphibians and birds, and their delicate mouthparts can even pierce the thick skin of reptiles (17, 30, 36). Many are of great importance however, because they have a high preference for biting humans (36).

Mosquitoes have greatly affected the course of human history, and continue to do so today. It's estimated that over a million people a year die from malaria and other mosquito-borne diseases (30, 36). These detrimental effects on human health are most prevalent in developing regions and the tropics. They can cause chronic debilitation in people due to the diseases they transmit, often straining the already scarce resources of health services in underdeveloped regions and reducing human productivity overall (36). This serves to perpetuate economic hardship in these regions of the world. The World Health Organization (WHO) calculates the economic cost of specific diseases using the disability adjusted life years (DALY)

index, which can be defined as the annual sum of the years of economically productive life lost due to disability and premature mortality in the population (37). Insect-borne diseases account for eight of the fifteen worst infectious diseases or disease clusters (http://www.who.int/healthinfo.statistics/gbdwhoregiondaly2002.xls). Malaria is fourth on the list (46.5 million DALYs), behind respiratory infections (94.6 million DALYs), HIV/AIDS (84.5 million DALYs), and diarrhoeal diseases (62 million DALYs), but well ahead of tuberculosis (35 million DALYs), and measles (21.5 million DALYs). Other mosquito-borne diseases (filariasis, dengue, and Japanese encephalitis) contribute an additional 7.1 million DALYs each year.

Aside from affecting human health, mosquitoes can have a tremendous impact on the economy. The United States, for example, spends hundreds of millions of dollars a year on mosquito control (36). The mere presence of large populations of mosquitoes can impact real estate values as well as tourist industries and other outdoor activities (30). Large populations of mosquitoes can cause severe irritation and blood loss to livestock and wildlife, resulting in decreased productivity, death, and economic losses (36) thus creating an interest in their ecology and behavior.

Mosquitoes belong to a specialized group of arthropods that require a blood meal for development of their eggs. At least 14 orders or families of arthropods (containing more than 15,000 species) have independently evolved the ability to feed on vertebrate blood (12). Female mosquitoes specifically require the protein from a blood meal to meet the high nutritional requirement for egg development (4, 17). The mean amount of blood that *Aedes* and *Anopheles* mosquitoes can ingest varies from two to four times the weight of the female (17).

How do they locate their sources of blood? In the early 1950s, Anthony Brown (7, 8) constructed human-shaped, steel tanks and used them to test different factors that might attract

mosquitoes. He found that his "robots" were most attractive when warmed to 37° C (normal human body temperature), exhaled CO_2, and were soaked in human sweat (7, 8). More recent studies have confirmed what Brown had discovered, showing that for most mosquitoes CO_2, heat, and moisture are the most important elements in locating a suitable host (17, 18, 20, 55). Cues such as these, however, may lead a mosquito to any warm-blooded animal, but species that exhibit extreme host specificity (such as feeding almost exclusively on humans or amphibians), must rely on more specific cues. Lactic acid, for example, is a chemical compound produced on the skin of humans. *A. aegypti* has been shown to be strongly attracted to lactic acid (18, 22). In contrast, *An. gambiae* was discovered to have a preference for biting humans on their feet. A Dutch researcher, Bart Knols, noted that human feet bore a similarity in odor to Limburger cheese, and showed that *An. gambiae* was strongly attracted to Limburger cheese (28). The common feature shared by the cheese and human feet was a bacterium used in cheese production, *Brevibacterium linens* (28). This bacterium is a close relative of *B. epidermis*, a bacterium known to reside between the toes of humans, and both produce a distinctive blend of volatile fatty acid esters, thus explaining the curious attraction to the cheese. *An. gambiae* has also been shown to be attracted to other compounds released from human skin such as 1-octene-3-ol and C4-C6 2-oxopropionic acids, as well as the previously discussed carbon dioxide and lactic acid (64). In 1996, Cork demonstrated that 1-octene-3-ol was only effective at attracting female mosquitoes in the presence of carbon dioxide, suggesting that it is the detection of a complex mixture of odors that is responsible for this insects' extreme host specificity (18, 64).

Once a mosquito does find a host, it faces the even more difficult task of manipulating the host's hemostatic system in order to facilitate obtaining a blood meal. Mosquitoes must localize a suitable blood vessel and begin ingestion as soon as possible, before rejection or

detection by the host, in order to maximize the possibility of escaping with the blood meal and her life. To facilitate this, mosquitoes have evolved sophisticated cocktails of pharmacologically active salivary reagents that function as platelet aggregation inhibitors, anticoagulants, immunomodulatory agents, and vasodilators (2, 3, 12, 47, 48, 59). Saliva containing these compounds is injected into the host whenever the mosquito probes or bites looking for a target blood vessel (16, 29, 45, 49). The following discussion will focus on *A. aegypti* as this species accounts for most of the work on mosquito saliva done to date and is the most common mosquito pest worldwide (3, 18-20, 42, 43). Differences between *A. aegypti* and the other species used in the present study will be addressed later.

The first group of compounds I will discuss are the platelet aggregation inhibitors. Under normal conditions, platelets in the blood respond to wound-generated signals (including ADP, thrombin, and exposed collagen) by becoming activated and aggregating to form a platelet plug, which obstructs the damaged vessel and prevents further blood loss (16). Activated platelets also degranulate and release additional ADP and platelet activating factor (PAF), as well as serotonin and thromboxane A2. These molecules recruit additional platelets to the platelet plug, and the latter two stimulate vasoconstriction. Activation of platelets also results in changes in the surface charge of the cell membrane, which provides a surface for assembly of enzyme complexes involved in the clotting cascade. Inhibition of these platelet responses is essential if the mosquito is to obtain a complete blood meal. In *A. aegypti*, platelet aggregation inhibitors include the enzyme apyrase, the D7 protein family, and the enzyme PAF esterase (17, 21, 25, 44).

Insect apyrases inhibit platelet aggregation (13, 26, 46) by the hydrolyzing adenosine di- and triphosphates that are released from injured cells or from activated platelets (15, 21, 49). By

inhibiting formation of the platelet plug, the mosquito effectively ensures continued blood flow, which benefits the mosquito by allowing it to spend less time on the host to complete the meal. Several studies have shown that higher levels of apyrase activity are directly correlated with decreased probing time during feeding (46, 47, 49, 53).

Other platelet aggregation inhibitors are members of the D7 protein family. Even though D7 proteins are some of the most abundantly expressed salivary proteins in blood feeding Diptera (12, 57), their function was only recently discovered. The D7 salivary family of proteins is distantly related to the odorant-binding protein superfamily (12). The physiological functions of this protein superfamily are not completely understood; however, the most widely accepted hypothesis suggests they facilitate the perception of chemical stimuli by binding odorant molecules (which tend to be small and hydrophobic), and transport them to odor receptors on the surface of olfactory neurons in an aqueous environment (40, 64). Calvo et al. (12) recently showed that D7 functions to bind amines including serotonin, histamine, and norepinepherine with high affinity. By sequestering these amines, D7 serves as both a platelet aggregation inhibitor and a vasodilator. In addition, by binding host amines, D7 serves to reduce the pain that would be felt when a female mosquito begins the probing process (12). This benefits the mosquito by helping her to quickly escape alive and unnoticed with the blood meal. A third platelet aggregation inhibitor that aids mosquitoes is the enzyme platelet aggregation factor esterase (PAF esterase). This enzyme aids the mosquito by destroying the platelet aggregating factor (PAF) released from activated platelets (59).

The next group of compounds to consider is the anticoagulants. In *A. aegypti* the main anticoagulant specifically inhibits activated Factor X (FXa) (41, 54). This anticoagulant belongs to a family of proteins called serine protease inhibitors (Serpins). These are important because

FXa, and indeed most clotting factors, are serine proteases. As discussed above, the D7 protein family may also contribute to the anticoagulant activity of saliva, by preventing assembly of enzyme complexes needed to generate the clotting cascade, on the surface of activated platelets. The function of anticoagulants in mosquito saliva is not as obvious as it might appear at first glance, as it takes less time for the insect to feed than it does for the clot to form. It is likely that saliva is mixed with the blood during feeding, and the anticoagulant activity prevents blood from clotting in the mouthparts and the midgut. This could help the mosquito to clean blood from the mouthparts after the meal, and it might improve digestibility of the (unclotted) blood meal.

The final group of compounds we will consider are the vasodilators. These play an important role in helping the mosquito to find a blood vessel, which can occupy less than 5% of the volume of the hosts' skin (16). In *A. aegypti* saliva, this role is filled by the peptide sialokinin (44). It benefits the mosquito by increasing the size or diameter of the target blood vessel, making it easier for the mosquito to find, and by enhancing the rate of blood flow to the feeding mosquito, thus decreasing the time needed to complete the meal (14). By reducing the contact time with the host, vasodilators reduce the opportunity for the host to detect the mosquito and kill it.

Recently, characterization of salivary mRNAs has revealed that there are many other proteins or peptides, most of unknown function, present in the saliva of mosquitoes (54, 59). While these proteins/peptides may currently have a unknown function, many of them undoubtedly serve as potential antigens (41, 42), such as the antigen 5 protein family members for example (59). These antigens may prove to have a significant role in driving host immune responses to mosquito biting.

It is also important to note that while members of the genera *Aedes* and *Anopheles* share some of these proteins, there are several major differences worth mentioning. For example, *Anopheles* species have a lower apyrase level than do *Aedes* species (3). They also lack the anticoagulant anti-Factor Xa (58), instead having a different protein, anophelin, with antithrombin activity (3, 58, 60). Thrombin is a serine protease that catalyses the final step in the coagulation cascade, converting fibrinogen to fibrin (16, 60). It is also responsible for inducing platelet aggregation in vertebrates, using a different active site (exosite) on the protein surface than the catalytic exosite responsible for fibrinogen cleavage (16). Anophelin works against both components of the hosts' hemostatic defenses by blocking both exosites, thereby inhibiting both thrombin-induced platelet aggregation (16, 58) and the clotting cascade. The inhibition of platelet aggregation may help compensate for the low levels of apyrase activity in *Anopheles* species (16, 60).

Another significant difference is the absence in *Anopheles* of a tachykinin peptide vasodilator, which is replaced by a salivary peroxidase that is a member of the myeloperoxidase gene family (50). This enzyme acts as a vasodilator by destroying catecholamines such as serotonin and norepinepherine, which normally function as endogenous vasoconstrictors (50). This destroys the host's ability to constrict blood vessels (at the point where saliva is introduced), resulting in a slow but persistent vasodilation.

Some components of the saliva serve as antigens, stimulating the hosts' immune system to start producing antibodies in a process called seroconversion (1). Salivary proteins are also taken up via pinocytosis by macrophages or dendritic cells, which function as antigen-presenting cells (APCs). These proteins are proteolytically processed, and peptide fragments are bound to major histocompatability complex type II (MHC II) and presented on the surface of the cells (1,

27). Subsequently the APCs migrate to nearby lymph nodes, where the peptide/MHC II complex activates specific $CD4^+$ T cells (T-helper cells). Activated T-helper cells leave the lymph nodes and secrete factors (cytokines) that activate macrophages, neutrophils, and other phagocytic cells, which then eliminate extracellular antigens (1, 27). T-helper cells also function in activating B-cells to produce antibodies that bind to foreign extracellular material, inactivating proteins and pathogens and accelerating their elimination by phagocytic cells (1, 27).

This process of seroconversion is well known to occur in the hosts of mosquitoes (3, 32, 62). Every time a mosquito bites its' host, it injects saliva that is responsible for the skin reactions hosts exhibit post-exposure (9, 11, 27, 49). Five stages of clinical reactivity typically occur in normal individuals after repeated exposure (3, 29, 41, 42, 44). It is important to note that the development of each stage is exposure-dependent and not age-dependent. It may be possible for a younger child who has been heavily exposed to mosquito bites to be at a more advanced stage than an adult who is only rarely exposed to bites (3, 29, 38). The first stage, in immunologically naïve individuals, is when no clinical reactivity is seen. The second stage involves a delayed type III hypersensitivity response that begins to express itself as a skin wheal-and-flare after 24 hours. The term "wheal" refers to a raised, reddened area of skin, and flare indicates that the margins of the wheal tend to extend in amoeboid-like projections. Stage three is characterized by type I and III hypersensitivity responses where skin papules are present 10-15 minutes after the bite with additional bite marks being visible after 12 hours. The fourth stage results in an immediate response that generally subsides within a couple of hours after exposure. Finally, stage five is when the host becomes tolerant to the mosquito and no clinical response is seen. These clinical symptoms indicate underlying immune events triggered by salivary antigens. Delayed-type III hypersensitivity results from infiltration of immune cells (especially

macrophages, eosinophils, and T-cells), into the skin at the bite site. Immediate type I hypersensitivity is mediated by histamine release from mast cells, which is stimulated by the binding of certain antibody isotypes with salivary antigens. The entire process is driven by antibody isotype switching, where different levels of exposure stimulate B-cells to switch from producing IgE to IgM and, eventually, IgG type antibodies. Each of these antibody isotypes stimulates the immune system in different ways, resulting in the different clinical responses.

There are numerous antigens in the saliva of mosquitoes. Most work to date has focused on *A. aegypti*, where more than 20 polypeptides are visible on SDS-PAGE gels, and immunoblot techniques, using serum from mosquito-sensitized human patients, have shown that up to 8 of these serve as antigens (41, 42). Identified antigenic proteins include both D7 (43) and apyrase (44). These proteins have also been identified as allergens in other mosquitoes, including several Culex and Aedes species (43).

Previous studies have shown that seroconversion can have a negative effect on the fitness of a vector (3, 23, 25, 32, 34, 56). *Amblyomma americanum* ticks are rejected by guinea pigs (an unnatural host for this tick) by a mechanism that involves both antibody and cell-mediated immune responses (9-11). This rejection may depend on the long attachment time (10 or more days) involved in feeding by ixodid ticks, as time is available for the development of immune responses and recruitment of immune cells to the feeding site. It is interesting that when ticks feed on their natural hosts this rejection does not occur, due to the capacity of ticks to modulate immune responses in these hosts (6).

In contrast to the long feeding times (and so exposure to the host immune system) seen in ticks, blood-feeding insects complete their meal on time scales from a few minutes (many flies including mosquitoes) to 30-40 minutes in the case of triatome bugs. Is there any effect of host

seroconversion affecting insects that feed quickly? Evidence for such an adverse effect on insect feeding/fitness can be seen in a 2004 study that used sandflies as their model. Sandflies express a salivary protein called maxadilan (MAX) that, similar to the previously mentioned sialokinin, functions to dilate vertebrate blood vessels (34). The study used naïve mice and mice immunized against MAX or sensitized to the bites of the flies to demonstrate that the sand flies acquired a larger blood meal from naïve mice when compared to the other mice used in the study (34). This suggests that the process of seroconversion can result in the production of antibodies that may lead to a negative effect on the arthropods' fitness (34). This is due to the fact that the number of eggs the female will produce is directly proportional to the amount of blood taken (24, 39).

Few studies have addressed the effect of anti-mosquito antibodies and immune responses on mosquito fitness. Most of these studies were concerned with the development of vaccination strategies to reduce mosquito populations, and so hosts were often challenged in an unnatural manner. For example, it was shown that when *A. aegypti* mosquitoes fed on a host that had been repeatedly injected with *A. aegypti* extracts, the result was lowered fecundity, smaller blood meal volumes, increased mortality, and a reduced F_1 viability (25, 51, 56, 62). However, as mice were injected with extracts from whole mosquitoes, they were exposed to extraneous proteins not present in the saliva (42). In nature, mosquito hosts are only exposed to salivary antigens, not the mosquitoes gut, brain, or other organs. The lowered fitness could have been due to any antigen, not just a salivary antigen.

Does the suggestion that seroconversion can adversely effect the mosquito hold true for salivary antigens? A 1996 study using *An. stephensi* mosquitoes as the model organism clearly demonstrated that mice that were repeatedly exposed to bites by *An. stephensi* began producing

antibodies to the salivary gland apyrase as detected by western blot analysis (32). This study was significant because it showed that these antibodies were capable of inhibiting the catalytic activity of the apyrase. However, these antibodies had no affect on the mosquito feeding (32). Possible reasons for this lack of effect are discussed later in this thesis.

The above studies suggest that it is possible that host seroconversion has a fitness impact on mosquitoes, but this has not been well demonstrated by experimental studies. The picture is further complicated if we consider that, in Nature, hosts will be exposed to biting from several mosquito species. Cross-reaction between antigens from different mosquito species (41-44) suggests the possibility that exposure to one species may have a subsequent fitness impact on other species that feed on that host. This issue has not been explored at all in the literature.

In this thesis I present a series of experiments designed to test the hypothesis that host seroconversion can adversely impact mosquito fitness. To ensure that results apply to mosquitoes in general, I conducted experiments with four mosquito species. The mosquito species used in the present study are of major importance. The four species used were *Aedes aegypti*, *Aedes albopictus*, *Anopheles stephensi*, and *Anopheles gambiae*. *Aedes* species have a tropical/subtropical global distribution (17, 36) while *An. stephensi* can be found in Southeast Asia and *An. gambiae* can be found in Africa. *A. aegypti* and *A. albopictus* are both responsible for the transmission of viral diseases, more specifically, yellow fever, dengue, and encephalitis (36). *An. stephensi* and *An. gambiae* on the other hand are both responsible for the transmission of malaria (36).

SPECIFIC AIMS

In chapter two, I explore the hypothesis that seroconversion has an adverse effect on mosquito feeding and fitness. Over a four-week period, naïve mice were exposed weekly to feeding by either *Aedes aegypti*, *Aedes albopictus*, *Anopheles stephensi*, or *Anopheles gambiae*. Blood meal sizes, the number of eggs laid, and the number of those eggs that were viable were measured each week. To test for cross-reaction between species, on the fifth week mice were exposed to all four-mosquito species. Again, blood meal sizes, the number of eggs laid, and the number of those eggs that were viable were measured. I verified that seroconversion had occurred by using western-blot analysis.

To confirm the role of seroconversion, the experiment was repeated using B-cell knockout mice with the respective controls and using *Aedes aegypti* only. B-cell knockout mice are unable to seroconvert. If seroconversion is necessary for the development of fitness-reducing effects, we predict that these effects will not be observed when mosquitoes are fed on B-cell knockout mice.

CHAPTER 2

MEASURING THE EFFECT OF REPEATED EXPOSURE ON MOSQUITO BLOOD MEAL SIZE AND FECUNDITY

Salivary glands can be defined as organs that synthesize and secrete proteins that aid in the acquisition of a blood meal (52). They are also the most important organs in the mosquito with respect to disease transmission. The salivary proteins that these organs secrete are an important component in the mosquitoes' arsenal of weapons that aid them in obtaining a blood meal (3, 13, 21, 49, 56, 59). Several of these proteins also serve as antigens, eliciting an immune response from the host (45). When injected into the host by the mosquito, these antigens cause the host to start producing antibodies specific to those antigens (1), a process known as seroconversion. Seroconversion is well known to occur in the hosts of mosquitoes (3, 32, 62) and this process has been shown to have negative effects on the fitness of other arthropods, including ticks and sandflies (3, 23, 25, 32, 34, 56). However, few studies have addressed the effect of host seroconversion on mosquito fitness, and most of these have used an unnatural protocol of exposing hosts to extracts of whole mosquitoes (25, 56). No studies that I am aware of have examined the effect of host seroconversion on the volume of blood ingested, or on the number of eggs produced.

The objective of this study was to determine if repeated feeding by mosquitoes on the blood of sensitized mice had an effect on mosquito fitness, specifically the volume of their blood meals and their fertility and fecundity. The four species used in this study were *Aedes aegypti*, *Aedes albopictus*, *Anopheles stephensi*, and *Anopheles gambiae*. All four of these species are of major human importance; the *Aedes* species are responsible for transmitting viral diseases such as Dengue, yellow fever, and encephalitis (17, 36), while, the *Anopheles* species are responsible

for the transmission of malaria (17, 36). The goal of this study is to test the hypothesis that host seroconversion has a negative effect on blood feeding and reproductive potential (measured as egg production and egg viability) in the four species aforementioned.

Materials and Methods

Mosquito Maintenance

Aedes larvae were reared at 26 ±1° C and fed a diet of yeast torula, macerated rodent diet, and Lactalbumin at a 1:1:1 ratio, while *Anopheles* larvae were fed a ground TetraMin diet. Adults were maintained at 26 ±1° C and 75 ±5% relative humidity under a 14-hour light/10 hour dark cycle. Adults were maintained on a 10% sucrose solution. All species used in this study were reared under the same conditions.

Blood Feeding Mosquitoes

The mice used in this study were housed in the animal facility in the Biological Sciences Building on the University of Georgia Campus. They were maintained and handled in accordance with the guidelines and regulations for the care and use of animals for research purposes, according to a protocol approved by the Animal Care and Use Committee at UGA. Prior to blood-feeding, female BALB/c mice (The Jackson Laboratory, Sacramento, CA) were anesthetized by an intraperitoneal injection of 0.2 mg of xylazine (Webster Veterinary Supply, Sterling, MA) and 2 mg of ketamine (Fort Dodge Vet Supply, Fort Dodge, IA). For certain experiments, B-cell knockout mice (mice unable to produce antibodies) as well as controls were used (strains B6.129S2-*Igh-6*^{tm1Cgn}/J and C57BL/6J respectively). Individual mice were marked by ear tag for identification purposes.

To determine the effect of exposure to mosquito feeding, groups of four mice were exposed to one of the four mosquito species tested. Once a week for four consecutive weeks, twenty-five female mosquitoes, which had emerged 5-7 days previously and had not previously blood-fed, were allowed to feed on each of four mice. A new (not previously blood-fed) set of mosquitoes was used each week. Mosquitoes were placed into 400 mL beakers covered in cheesecloth that was secured with rubber bands. An anesthetized mouse was placed on top of each of the four beakers; and mosquitoes were allowed to blood-feed for 20-25 minutes (i.e., until mice began to recover from the anesthesia). In each of the initial four weeks, mice were exposed to only one of the four mosquito species (*Aedes aegypti, Aedes albopictus, Anopheles stephensi, or Anopheles gambiae*). The experiment was repeated for each mosquito species.

To test for cross-reactivity between mosquito species, on the fifth week groups of fifteen females of each of the four mosquito species were blood-fed on each of the four mice. The order in which the species fed was randomized.

Quantifying Blood Meal Sizes

Ten mosquitoes from each group of twenty-five were randomly selected and their midguts were removed by dissection. Each gut was placed into 1 mL of distilled water (diH_2O) and torn open to allow osmotic lysis of erythrocytes and release of the hemoglobin, vortexed, and then placed on ice. After collecting the 40 guts, and after ensuring each had ruptured and was uniformly mixed with the diH_2O, the samples were spun down for 5 min at 14,000 rpm. 200 μL of each sample was then placed in a well of a 96-well plate. These were read at λ=413nm on a microtiter plate reader (Spectra MAX 340, Molecular Devices, Sunnyvale, California) and the resulting optical densities were used in conjunction with the Beer-Lambert law to determine the amount of hemoglobin (which will be proportional to the size of the blood meal) each mosquito

had taken. An extinction coefficient of 524280 OD units·cm^{-1}·g^{-1} was used in these calculations (http://omlc.ogi.edu/spectra/hemoglobin/summary.html). In the fifth week, five individuals of each mosquito species were analyzed in the above manner.

Quantifying Number of Eggs Laid and Egg Viability

From each of the initial four groups of 25 mosquitoes, 10 were randomly selected to lay eggs. After blood feeding, mosquitoes were placed individually in oviposition chambers consisting of 3.8 cm lengths of 3.5 cm diameter cylindrical clear acrylic tubing, with the top covered with a metal mesh. This was then placed on a Kimwipe EX-L, folded into a 5 cm square to serve as a substrate for egg laying, in a 60 mm petri dish, and secured with a rubber band. 1 mL of water was added to each dish to keep the Kimwipe wet. These oviposition chambers were then placed in large, covered, plastic containers, with moistened paper towels on the bottom to maintain humidity, in the rearing room under the conditions previously described for mosquito rearing. The mosquitoes were given one week to lay their eggs and the resulting eggs were counted under a dissecting microscope using a hand-held cell counter. Eggs were stored for 10 days at room temperature to mature, then placed in plastic weigh boats and covered with 1.5 cm of diH_2O and were given one week to hatch. Eggs were monitored for hatching and fed over the course of the week. The resulting larvae were counted using a hand-held cell counter.

On week five, for each mouse five individual mosquitoes of each of the four species were picked at random, and egg production and viability were determined as described above.

Collecting Serum for Western Blot Analysis

On the day following the week five feeding, mice were anesthetized by an intraperitoneal injection of 0.2 mg of xylazine (Webster Veterinary Supply, Sterling, MA) and 2 mg of ketamine (Fort Dodge Vet Supply, Fort Dodge, IA). They were then euthanized using CO_2 and blood was

collected by intracardiac puncture using a syringe. This blood was allowed to clot overnight at 4° C. The samples were then spun down at 14,000 rpm for 5 min. The serum was removed with a micropipette and stored in cryogenic vials at -70° C.

Western Blot Analysis

Salivary glands from the 4 mosquito species used in this study were removed and stored in 20μL of a HEPES saline solution (NaCl 0.15 M, 10mM Hepes, pH 7.0) along with 2μL of protease inhibitors (Mini-Complete, GIBCO/BRL). A protein assay was conducted (Micro BCA Protein Assay Reagent Kit, Pierce) to determine the relative amounts of protein present in a pair of *Aedes* and *Anopheles* salivary glands. The results of this assay were used to ensure that the same relative amounts of protein (20 μg) were loaded into the gels. The glands were lysed using a tissue sonicator (Branson) and then 5.8 salivary gland pairs (SGP) of each *Aedes* species and 10 SGP of each *Anopheles* species were added to loading dye, boiled for 5 minutes, and loaded into a 10-20% pre-cast Criterion gradient gel (BioRad). The proteins were separated for ~3 hours at 80 volts at 4° C **(**running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS). The transfer was done using Immun-Blot PVDF Membrane (Bio-Rad) overnight at 4° C with 20 volts (transfer buffer: 25 mM Tris, 192 mM glycine, 15% methanol). After the transfer was complete, the PVDF membrane was washed one time (washing buffer: 0.1M NaCl, 0.01M Tris pH 7.5, 0.1% Tween 20). The membrane was then blocked (blocking buffer: .33 M NaCl, .033 M Tris pH 7.5, 0.33% Tween 20, 1:20 blotting grade dry milk) at room temperature on a plate rocker for 2 hours at room temperature. It was then washed 1x in the washing buffer and put into a hybridization oven bottle with the primary antibody produced by the mice diluted with blocking buffer, 1:500 for *Aedes* and 1:250 for *Anopheles* at 4° C. These were incubated overnight at 4° C on a plate rocker.

After this incubation, the membrane was washed 3x consecutively in the washing buffer, then 1x every 2 minutes for a total of 4 times, followed by a final 30 minute wash. The secondary antibody (anti-mouse IgG conjugated to horseradish peroxidase) was diluted 1:5000 in the blocking buffer, added to the membrane, and placed on a plate rocker for 1 hour at room temperature. The membrane was washed 3 times consecutively in the washing buffer, followed by four 2 minute washes. Finally, 1mL of LumiLight Western Blotting Substrate (Roche) was added to the membrane, which was wrapped in plastic film. 5 minutes were allowed to pass and the membrane was exposed using Kodak BioMax XAR Film for 20 sec.

Statistical Analysis

The unpaired *t* test was applied to find the value of statistical significance of the experimental results. Results (mg hemoglobin, egg numbers, and number of larvae) for each week of the experiment were compared to week 1 results (naïve mice) separately for each mouse, for an n=10/mouse (n=the number of mosquitoes assayed for either egg laying or dissection). To assess overall treatment effects, time points were also compared using the mean value for each mouse as a data point; in these analyses n=4.

Results

Effect of Repeated Host Exposure to Mosquito Feeding on Egg Production and Viability

When mice were exposed to five consecutive weekly bouts of blood feeding by mosquitoes, there was a progressive decrease in the in the number of eggs laid, the viability of those eggs (i.e. in the proportion hatching) and size of the blood meal taken. This effect was seen with all four-mosquito species tested.

Aedes aegypti sensitized mice

When *Aedes aegypti* fed on naïve (week 1) mice, they laid a mean (± standard deviation) of 88 ± 4.8 eggs (Figure 2.1). The number of eggs produced increased slightly but significantly, to 98.9 ± 6.2 eggs/female ($p = 0.032$), when mosquitoes fed on mice that had had a single previous exposure to mosquito biting (week 2 mice). Subsequently, the number of eggs produced declined with each successive week, such that egg production was significantly reduced, compared to feeding on naïve mice, on week 4 (73.8 ± 3.25 eggs/female, $p = 0.003$), and even more so on week 5 (47.6 ± 14 eggs/female, $p = 0.002$). Overall this represents a 46% decrease in egg production when *Aedes aegypti* mosquitoes fed on mice that had experienced four weekly exposures to biting by conspecific mosquitoes. The same pattern of weekly decline was seen in all four mice tested; the magnitude of the decrease ranged from 63% (mouse 3) to 30.5% (mouse 1), and it was statistically significant in all cases.

A similar pattern of weekly decreases was seen in the production of viable (hatching) eggs (Figure 2.2). In contrast to total egg production, the number of viable eggs did not increase in week 2 (week 1: 81 ± 3 eggs/female; week 2: 83.1 ± 8.7 eggs/female; $p = 0.676$). In subsequent weeks viable egg production declined significantly, such that by week 5 the number of larvae produced (30.5 ± 12.5) was only 38% of the number produced when mosquitoes fed on naïve mice. Again, this decline was seen with all four mice. The decline was due not only to a decrease in the total number of eggs produced, but also to a progressive decline in egg viability (Figure 2.3). Ninety-two ± 1.8% of eggs produced following feeding on naïve mice were fertile, but the proportion of viable eggs decreased gradually to 63 ± 11.7% ($p = 0.0126$) by week five.

Blood meals, measured as micrograms of hemoglobin ingested, were significantly increased on week 2 ($p = 0.005$) (Figure 2.4). Meal volumes taken from week 3 and 4 mice were

not significantly different than meals taken from naïve week 1 mice. Blood meals taken from week 5 mice were significantly smaller than meals taken from naïve mice ($p = 0.002$).

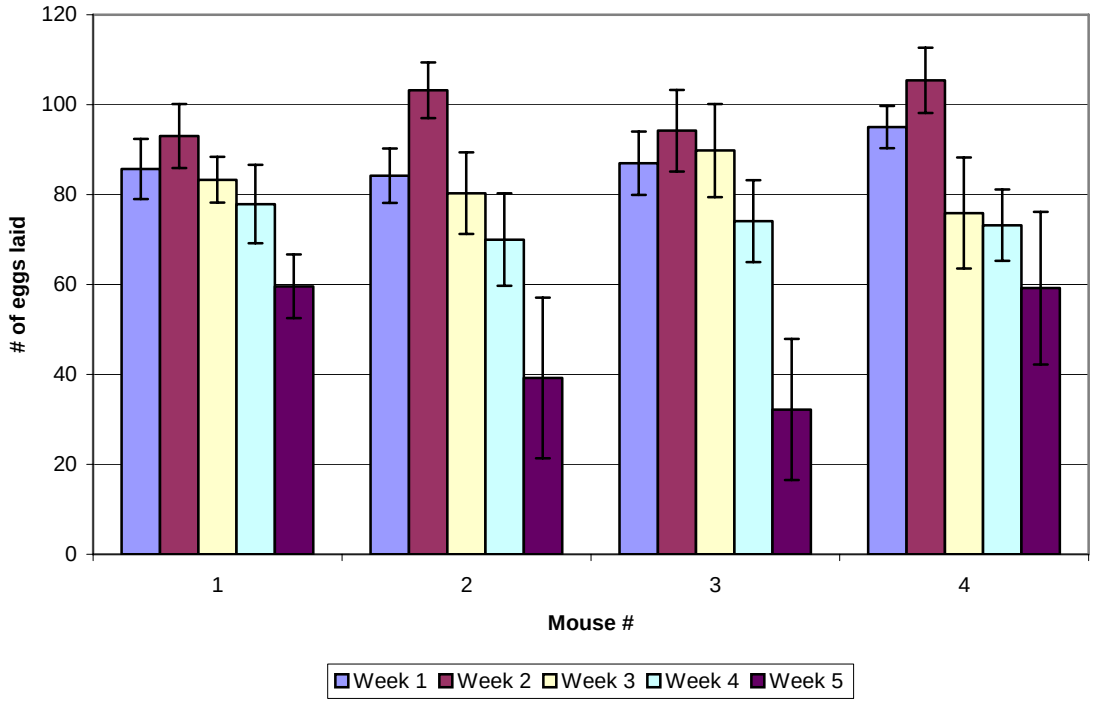

Figure 2.1: Effect of exposure to *Aedes aegypti* feeding on the number of eggs laid per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point).

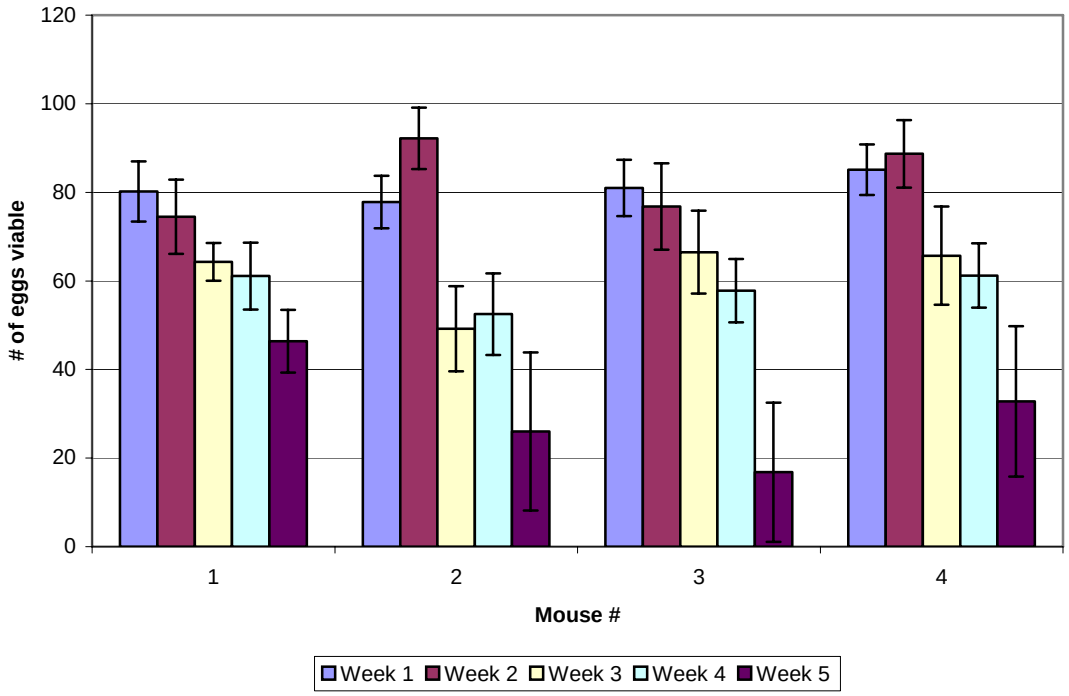

Figure 2.2: Effect of exposure to *Aedes aegypti* feeding on the number of eggs viable per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point).

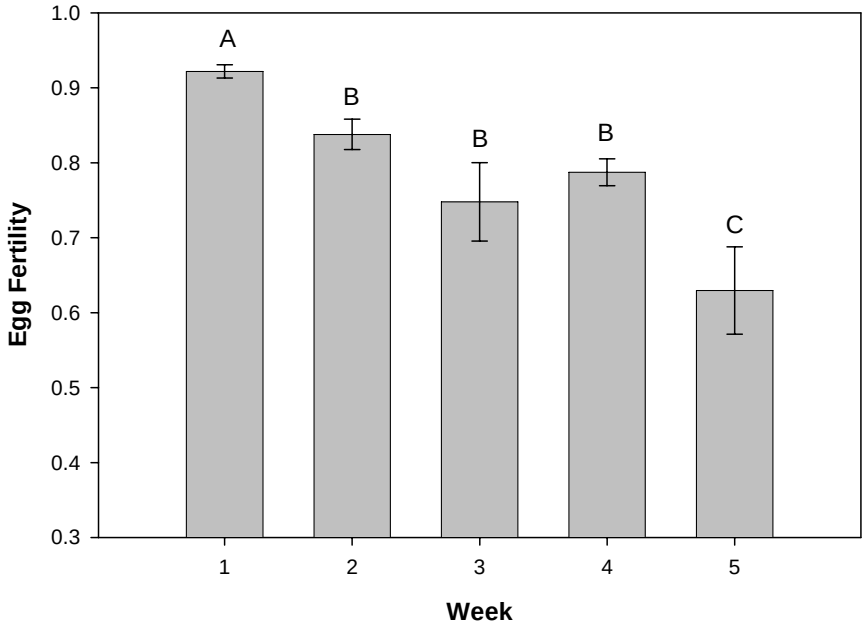

Figure 2.3: Proportion of eggs viable for mice sensitized to bites from *Aedes aegypti*. Error bars indicate ± one standard error (n=10 for each mouse/time point. Columns with different letters above them are significantly different ($p<0.05$, T-test compared to week 1 results).

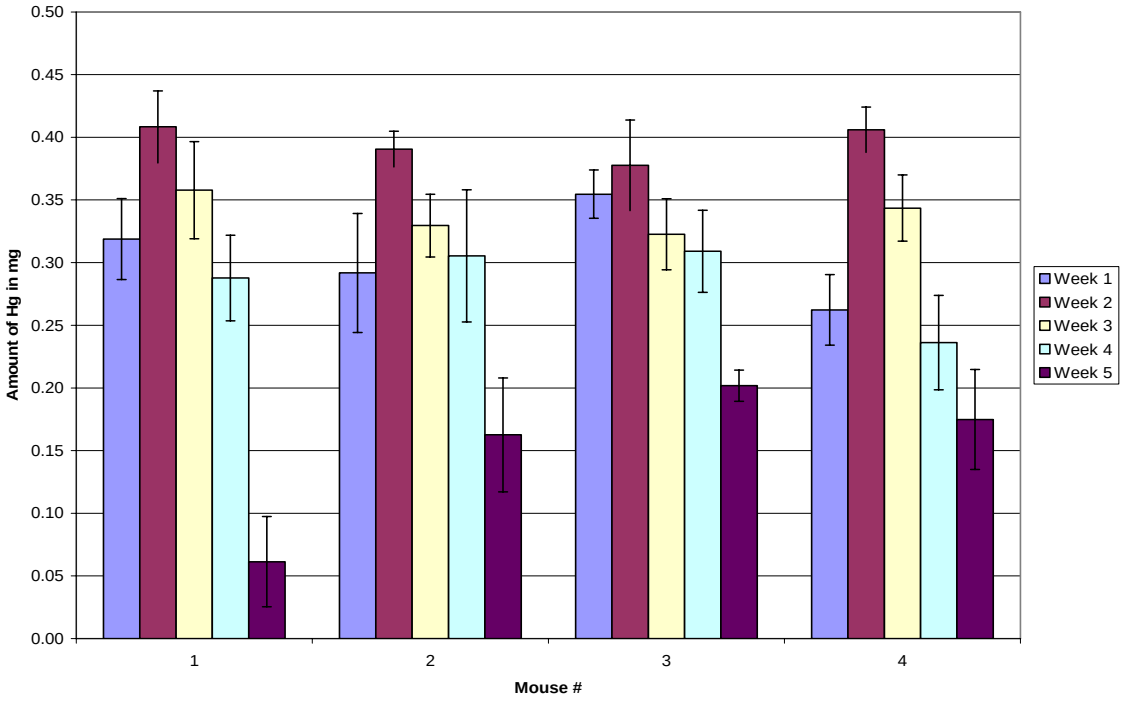

Figure 2.4: Effect of exposure to *Aedes aegypti* feeding on blood meal volume. Error bars indicate ± one standard error (n=10 for each mouse/time point).

Aedes albopictus sensitized mice

The pattern of progressively declining egg production and meal sizes, seen with *Aedes aegypti*, was even more strikingly developed when *Aedes albopictus* females fed on sensitized mice. *A. albopictus* fed on naïve mice (week 1) laid a mean of 112 ± 1.7 eggs (Figure 2.5). Subsequently, the number of eggs produced declined each successive week. For week 4, the mean number of eggs produced decreased to a mean of 74 ± 5.7 eggs laid ($p<0.001$). The pattern continued in week 5, where the mean number of eggs produced was reduced even further to 24.4 ±17.8, ($p<0.001$). This represents a 79% decrease in egg production when *A. albopictus* mosquitoes fed on mice that had experienced four weekly exposures to biting by conspecific mosquitoes. The same trend of weekly decline was seen in all four mice tested; the magnitude of the decrease in egg production ranged from 100% (mouse 1) to 63.4% (mouse 3), and it was statistically significant in all cases. A corresponding trend of weekly decreases was seen in the production of viable eggs (Figure 2.6). The number of fertile eggs decreased from a mean of 108 ± 1.52 (96.7% of total eggs) when mosquitoes fed on naïve mice to a mean of 95 ± 5.3, ($p=0.003$) for week 2. The pattern continued for each successive week, with the mean on week 5 reduced to just 14 ± 10.4, (56.5% of the eggs laid) ($p<0.001$). The decline in numbers of viable eggs was not due only to a decrease in the total number of eggs produced, but also to a progressive decline in egg viability (Figure 2.7).

Blood meals showed a steady decrease throughout the course of the experiment, with an average blood meal of 0.407 mg hemoglobin on week 1, decreasing to 0.345 mg for week 2 ($p<0.001$). This trend continued for weeks 3, and 4 until on week 5 blood meals had a mean of only 0.047 mg hemoglobin ($p<0.001$) (Figure 2.8). This represents a net 86% decrease in the mean amount of blood taken.

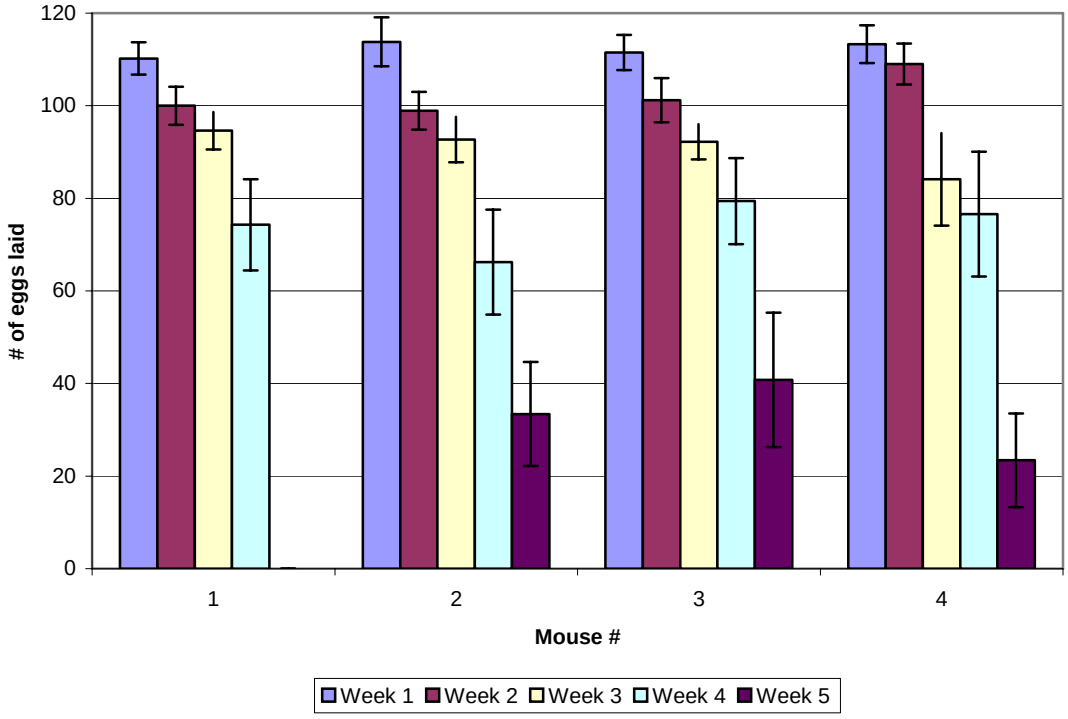

Figure 2.5: Effect of exposure to *Aedes albopictus* feeding on the number of eggs laid per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point). There were no eggs produced from mouse one on week 5.

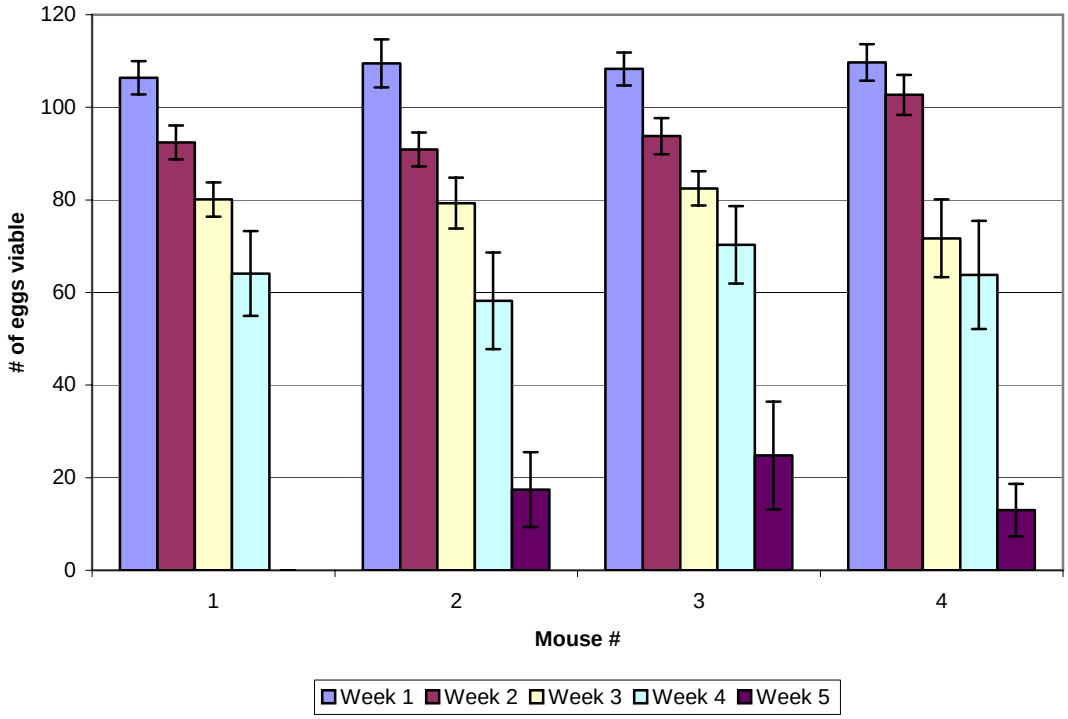

Figure 2.6: Effect of exposure to *Aedes albopictus* feeding on the number of eggs viable per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point). There were no eggs produced from mouse one on week 5 and so there were none viable.

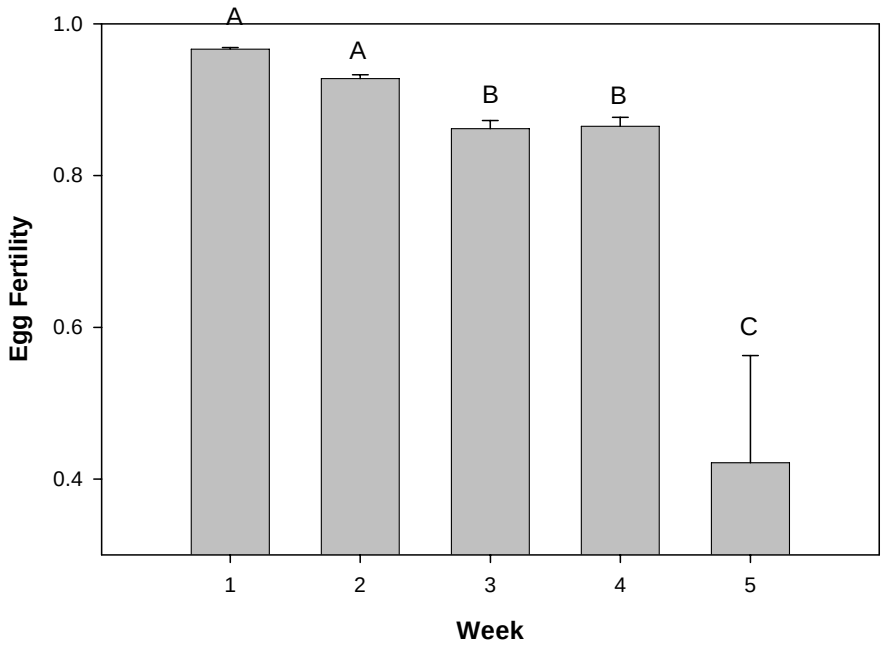

Figure 2.7: Proportion of eggs viable for mice sensitized to bites from *Aedes albopictus*. Error bars indicate ± one standard error (n=10 for each mouse/time point). Columns with different letters above them are significantly different ($p<0.05$, T-test compared to week 1 results).

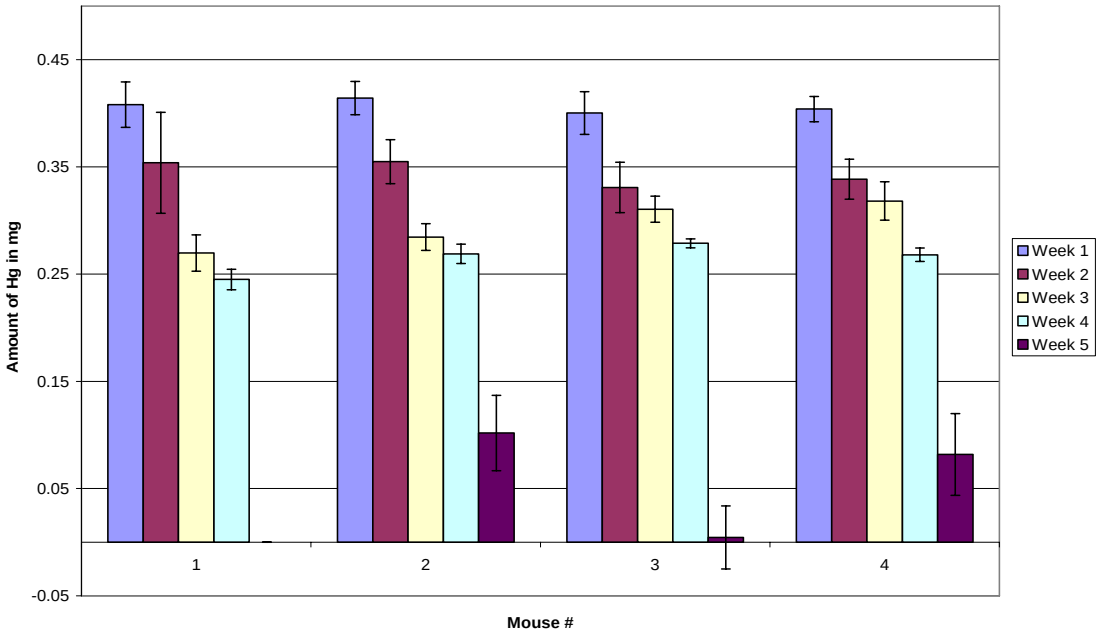

Figure 2.8: Effect of exposure to *Aedes albopictus* feeding on blood meal volume. Error bars indicate ± one standard error (n=10 for each mouse/time point) Mosquitoes did not feed very well on mouse one during week 5 and so there were none to dissect to measure blood meal amount.

Anopheles stephensi sensitized mice

When *Anopheles stephensi* fed on naïve mice the mosquitoes laid a mean of 119 ± 3.8 eggs. With each successive week, the mean number of eggs dropped slightly but significantly (Figure 2.9). Week 2 averaged 105 ± 5.7 eggs, ($p=0.008$), week 3 decreased further to a mean of 82 ± 11.6 eggs, ($p<0.001$), and week 4 had a mean of 45 ± 4.99 eggs, ($p<0.001$). On week 5, the mean number of eggs produced had dropped to 28 ± 9.89, ($p<0.001$). Overall, this represents a 76% in egg production. This weekly decline was seen in all 4 mice tested; the magnitude of the decrease of egg production ranged from 87% (mouse 3) to 69% (mouse 4), and it was statistically significant in all cases.

As with the other mosquitoes studied, weekly decreases were also seen in the number of viable eggs for mice sensitized to bites from *An. stephensi* (Figure 2.10). As was seen with the two *Aedes* species, the decline in numbers of viable eggs was due to a decrease in the total number of eggs produced, in combination with a progressive decline in egg viability (Figure 2.11). When mosquitoes fed on naïve mice, 81.5% of the eggs laid were viable. This percentage declined significantly through weeks 2, 3, and 4, so that by week 5 an average of only 13.4 ± 5.5 fertile eggs (47.8% of the total eggs laid) were viable. This represents a net reduction of 86.2% ($p<0.001$) in the production of fertile eggs, compared to feeding on naïve mice

As seen in the other two previously described mosquitoes, mice sensitized to bites from *An. stephensi* showed a decrease in blood meal volumes over the 5 week period. Week 1 blood meals contained a mean of 0.374 mg hemoglobin. Statistically significant decreases were not seen until week 4, which had a mean of 0.234 mg hemoglobin ($p=0.002$), and week 5 which had a mean of only 0.186 mg hemoglobin ($p<0.001$) (Figure 2.12).

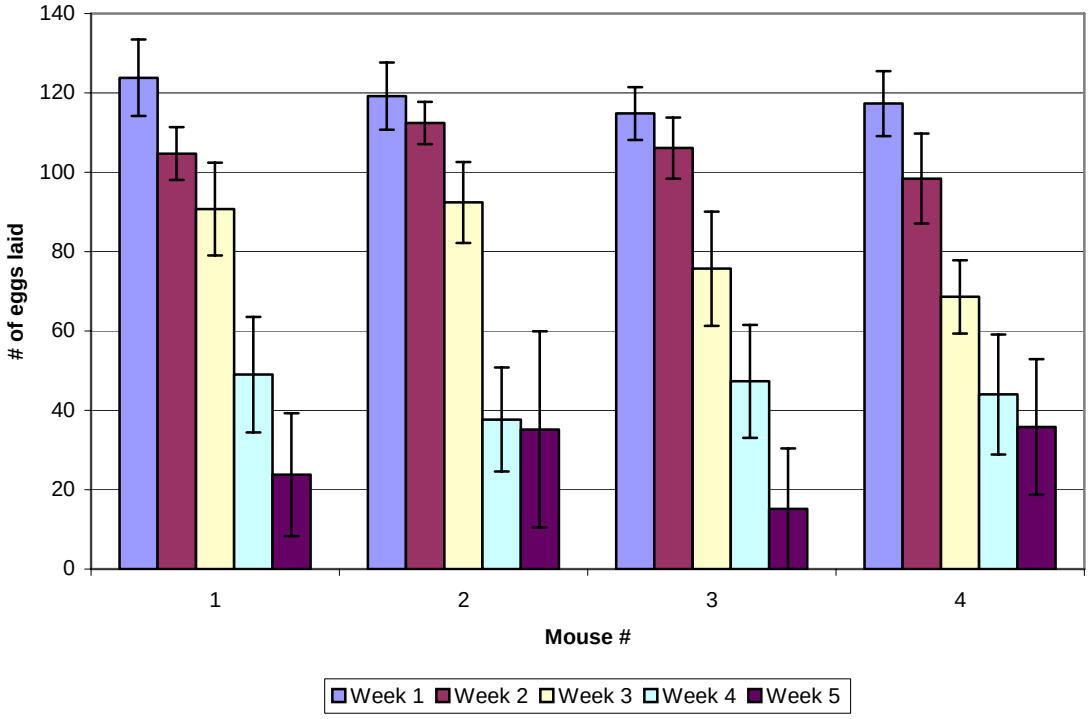

Figure 2.9: Effect of exposure to *Anopheles stephensi* feeding on the number of eggs laid per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point).

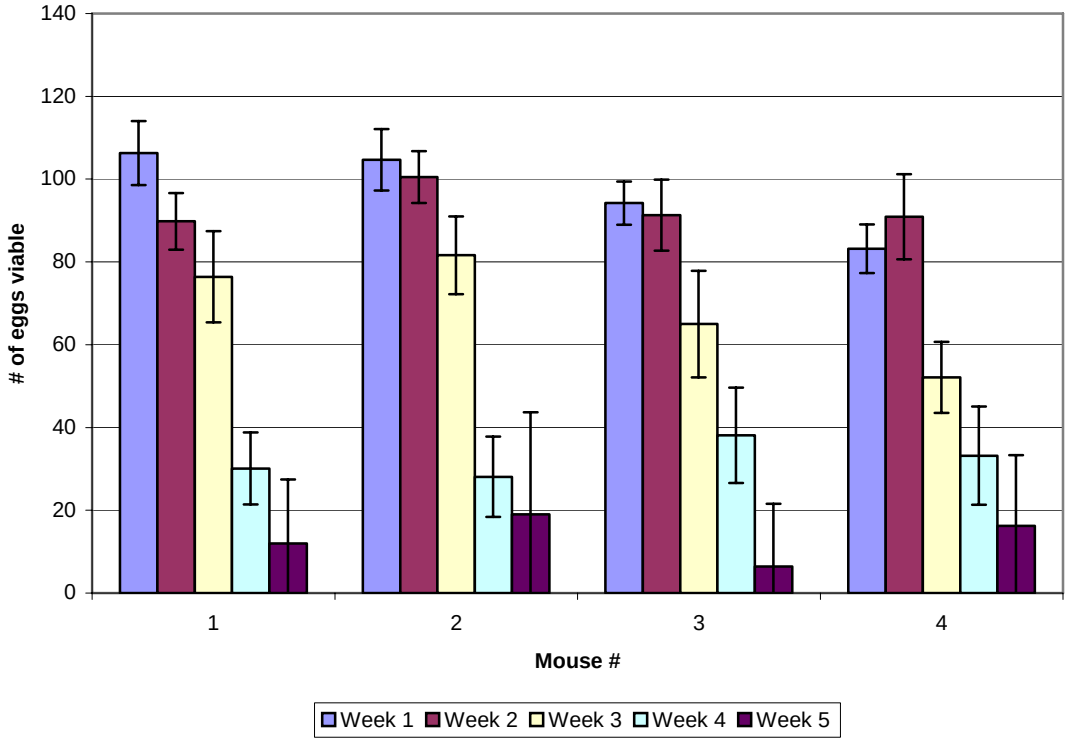

Figure 2.10: Effect of exposure to *Anopheles stephensi* feeding on the number of eggs viable per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point).

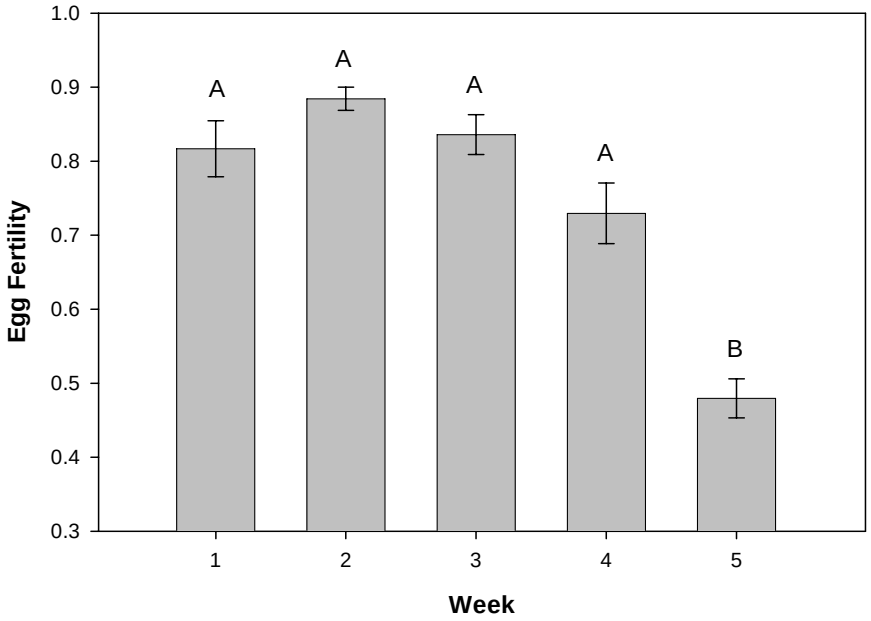

Figure 2.11: Proportion of eggs viable for mice sensitized to bites from *Anopheles stephensi*. Error bars indicate ± one standard error (n=10 for each mouse/time point). Columns with different letters above them are significantly different ($p<0.05$, T-test compared to week 1 results).

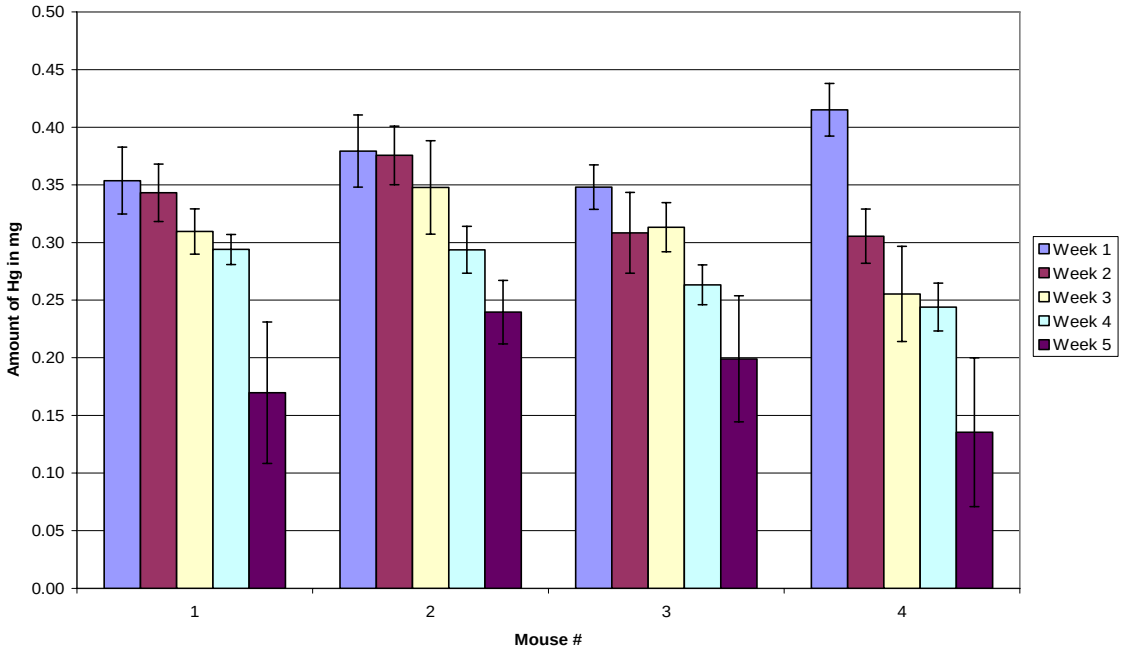

Figure 2.12: Effect of exposure to *Anopheles stephensi* feeding on blood meal volume. Error bars indicate ± one standard error (n=10 for each mouse/time point).

Anopheles gambiae sensitized mice

When *An. gambiae* fed on naïve mice (week 1), they laid a mean of 99 ± 8.11 eggs. Each consecutive week showed a statistically significant decrease in egg production. Week 3 had a mean of 77 ± 8.58 eggs ($p=0.009$). Week 4 continued the trend with a mean of 55 ± 15.4, ($p=0.002$) and on week 5 a mean of only 39 ± 2.20, ($p<0.001$) eggs were produced (Figure 2.13). From week 1 to week 5, there was a 61% reduction in the numbers of eggs laid. The same pattern was seen in all 4 mice tested: the magnitude of the decrease of egg production ranged from 62% (mouse 1) to 57% (mouse 4), and it was statistically significant in all cases. (Week 2 data were not included in this analysis, as we inadvertently used unmated females (which will not oviposit) for the blood feeding).

A similar trend was seen in the number of eggs that were viable over the course of the 5 weeks. Similarly to the other mosquito species studied, the decline in numbers of viable eggs was due to a decrease in the total number of eggs produced, in combination with a progressive decline in egg viability (Figure 2.15). Week 1 blood meals produced a mean of 92 ± 8.21 viable eggs, or 93% of the total eggs laid. Week 3 blood meals produced a mean of 58 ± 10.1 eggs ($p=0.002$). Weeks 4 and 5 continued the decrease with means of 37 ± 11.34, ($p<0.001$) and 18 ± 4.56, ($p<0.001$) respectively (Figure 2.14). When compared to week 1 that had a 93% viability rate, week 5 had only a 45% viability rate. That is a 48% reduction in the number of fertile eggs over the 5-week period.

The size of the blood meals taken by female *An. gambiae* showed the same trend as the other 3 previously described species. When they fed on naïve mice (week 1), they ingested an average of 0.269 mg of hemoglobin. There was a steady decrease in all 4 mice, but this was not

statistically significant (p=0.003) until week 5, when blood meals contained a mean of 0.157 mg of hemoglobin (Figure 2.16).

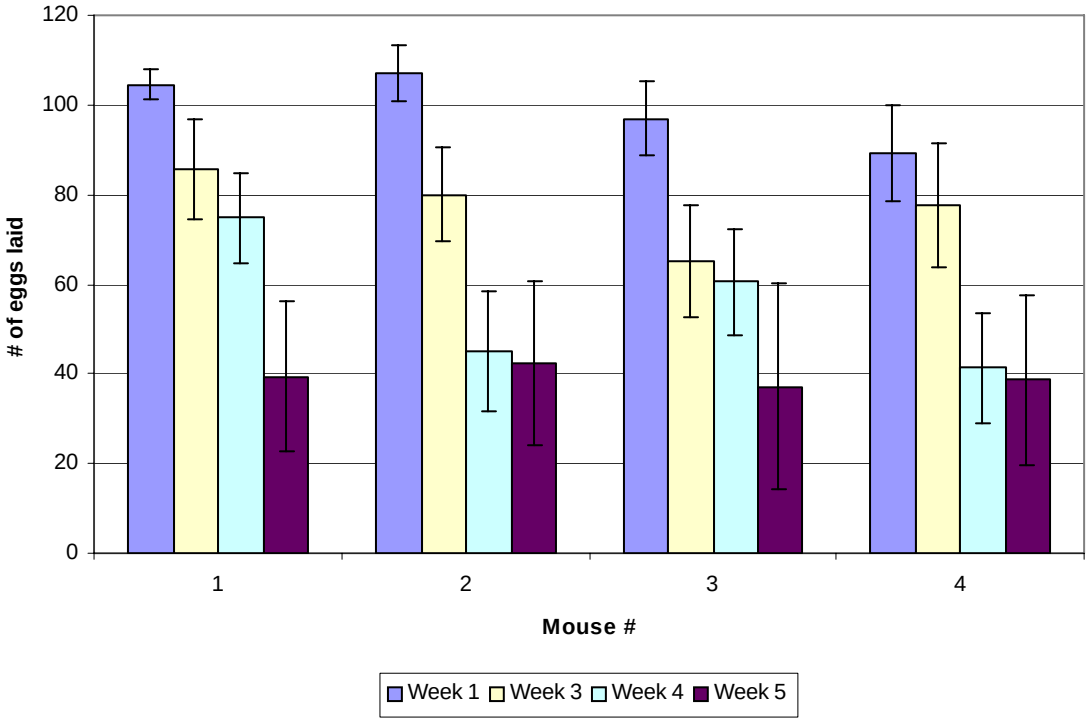

Figure 2.13: Effect of exposure to *Anopheles gambiae* feeding on the number of eggs laid per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point) Week 2 data were not included in this analysis, as we inadvertently used unmated females (which will not oviposit) for the blood feeding.

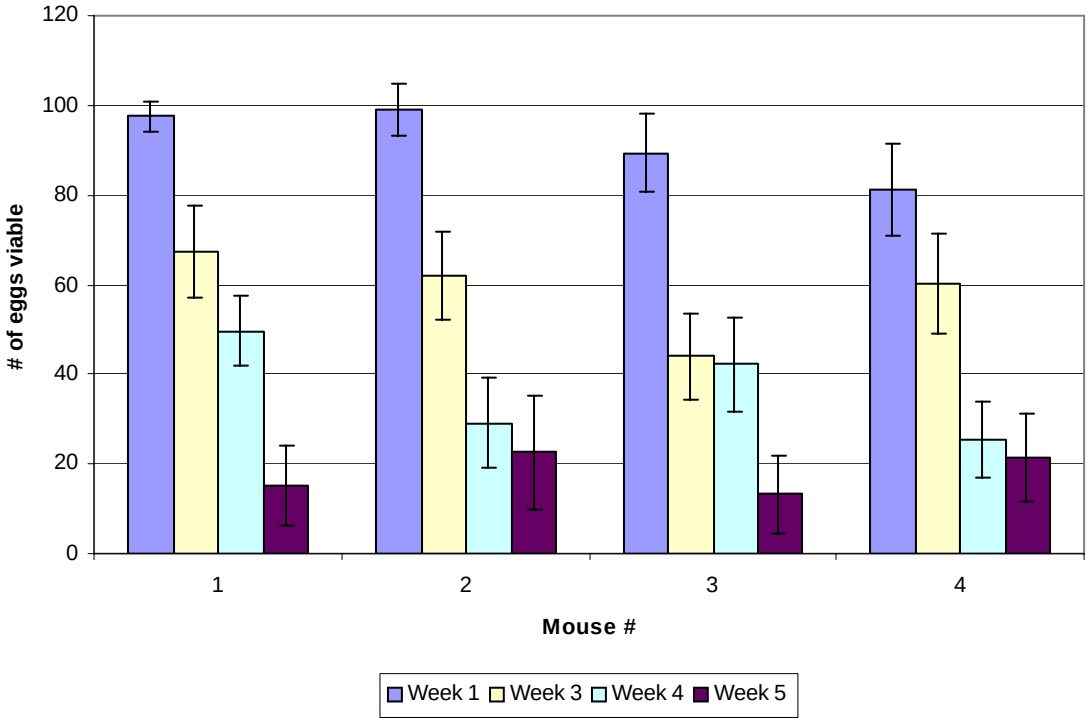

Figure 2.14: Effect of exposure to *Anopheles gambiae* feeding on the number of eggs viable per blood meal. Error bars indicate ± one standard error (n=10 for each mouse/time point) Week 2 data were not included in this analysis, as we inadvertently used unmated females (which will not oviposit) for the blood feeding.

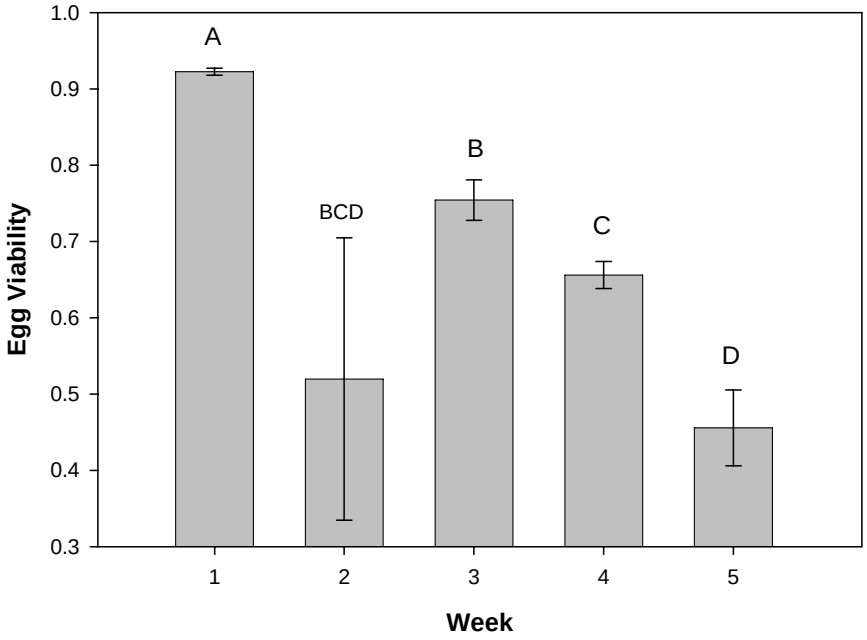

Figure 2.15: Proportion of eggs viable for mice sensitized to bites from *Anopheles gambiae*. Error bars indicate ± one standard error (n=10 for each mouse/time point). Columns with different letters above them are significantly different ($p<0.05$, T-test compared to week 1 results).

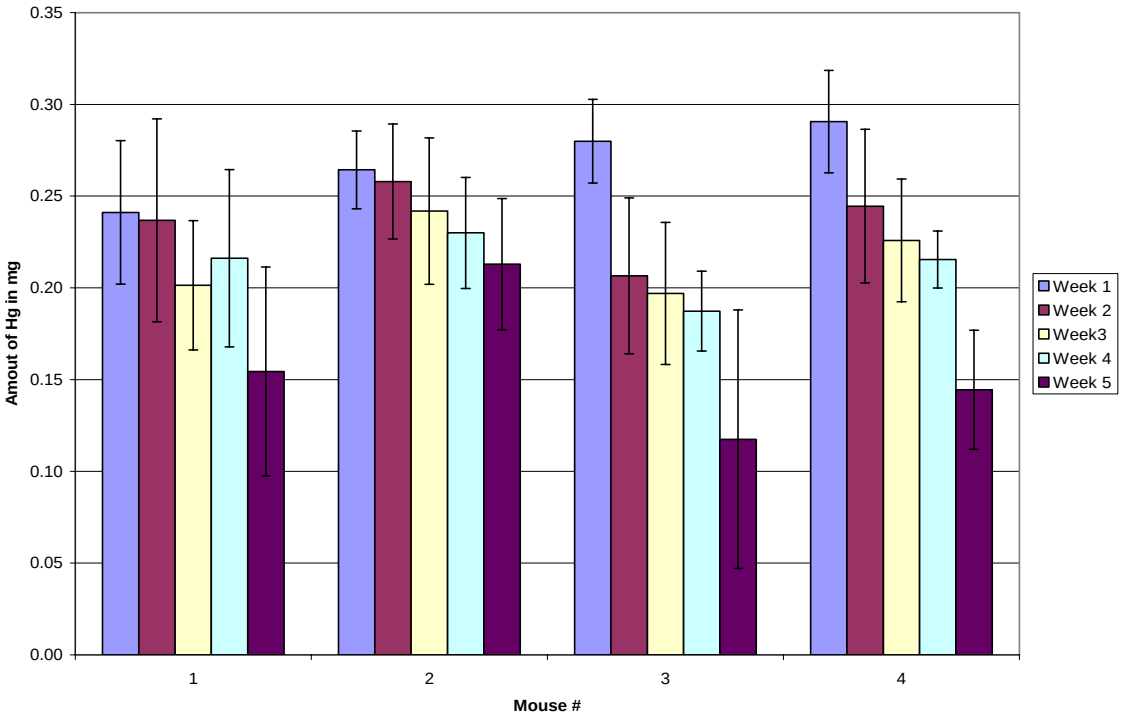

Figure 2.16: Effect of exposure to *Anopheles gambiae* feeding on blood meal volume. Error bars indicate ± one standard error (n=10 for each mouse/time point).

Cross-Reactivity Between Species

Mice, sensitized to *Aedes aegypti* salivary antigens by four weekly exposures, were used (on week 5) to feed *Aedes albopictus*, *Anopheles stephensi*, and *Anopheles gambiae* mosquitoes.

For *A. albopictus*, total egg production was significantly reduced, to only 53 ± 18.3 eggs/female, compared to a mean of 112.2 ± 1.7 eggs when *A. albopictus* fed on naïve mice (Figure 2.17). Production of viable eggs was reduced even more dramatically, from 108 ± 1.52 to only 36 ± 13.7, ($p<0.001$) (Figure 2.18), a net reduction of 67%. The sensitized mice also affected *A. albopictus*' ability to get a blood meal. When *A. albopictus* fed on naïve mice, they ingested a mean of .407μg of hemoglobin (Figure 2.8). However, after feeding on mice that were sensitized to *A. aegypti* salivary antigens, the mean volume of their blood meals was reduced by 54% to 0.186μg of hemoglobin (Figure 2.19).

On the other hand, sensitization to *A. aegypti* saliva had much less of an effect on *An. stephensi*, with egg production averaging 94 ± 10.5 eggs/female, compared to a mean of 119 ± 3.8 when feeding on naïve mice. Sensitization to *A. aegypti* saliva had no effect on egg production by *An. gambiae* females, with a mean of 99 ± 8.28 eggs compared to 99.4 ± 8.1 eggs when blood meals were taken from naïve mice.

Host sensitization to *A. aegypti* saliva had no significant effect on feeding by either *Anopheles* species. On week1, when feeding on naïve mice, *An. stephensi* and *An. gambiae* gut contents indicated a mean meal size of 0.374 mg and 0.269 mg of hemoglobin respectively. After feeding on mice sensitized to *A. aegypti*, *An. stephensi* and *An. gambiae* guts contained a mean of .374 mg and .337 mg of hemoglobin respectively (Figure 2.19).

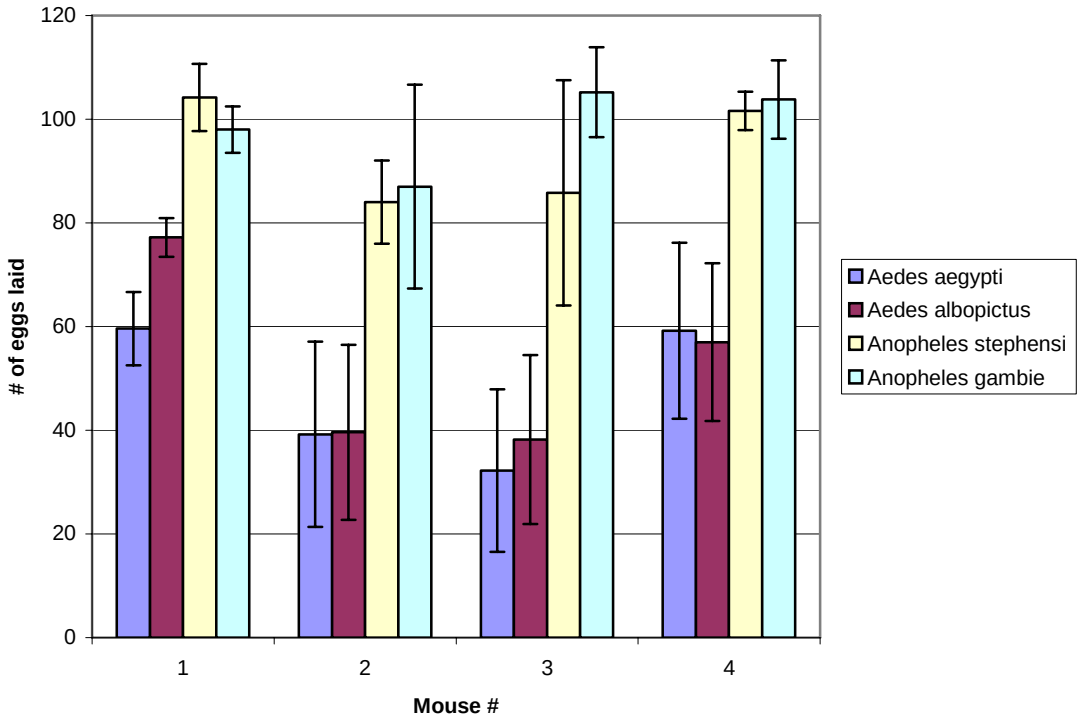

Figure 2.17: Effect of exposure to all 4 species used on number of eggs laid for mice sensitized to bites from *Aedes aegypti*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

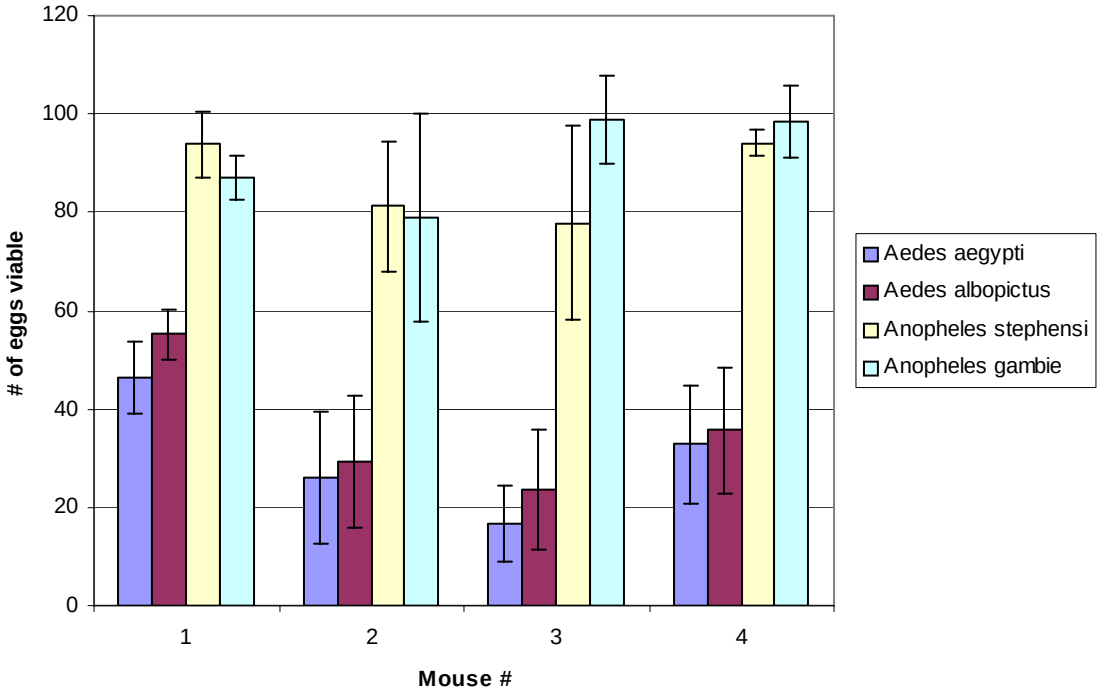

Figure 2.18: Effect of exposure to all 4 species used on number of eggs viable for mice sensitized to bites from *Aedes aegypti*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

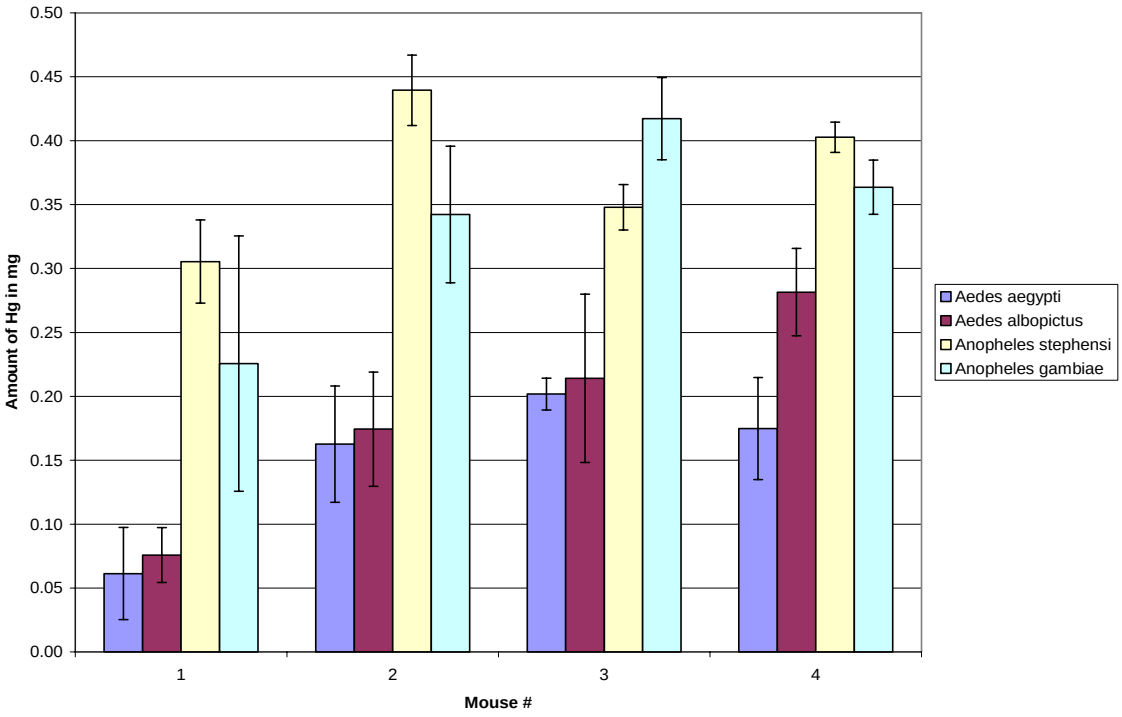

Figure 2.19: Effect of exposure to all 4 species used on blood meal volume for mice sensitized to bites from *Aedes aegypti*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

Mice, sensitized to *Aedes albopictus* salivary antigens by four weekly exposures, were used to feed *Aedes aegypti*, *Anopheles stephensi*, and *Anopheles gambiae* mosquitoes. *A. aegypti* egg production was inhibited following feeding on *A. albopictus* sensitized mice. These females laid and average of 67 $\pm$ 18.8 eggs, compared to 88 $\pm$ 4.8 eggs produced from feeding on naïve mice. This 24% reduction was not as pronounced as the reciprocal effect of sensitization to *A. aegypti* on *A. albopictus* egg production, which was reduced by 52% (Figure 2.20). Production of viable eggs was also reduced (by 29.6%) for *A. aegypti* feeding on mice sensitized to bites from *A. albopictus*, from 81 $\pm$ 3.04 to only 57 $\pm$ 17.7 fertile eggs, (Figure 2.21). This reduction in fertile egg production was not due to an inhibition of feeding, as blood meals on sensitized mice contained a mean of .355 μg of hemoglobin, which was an increase over the mean meal size of .307μg hemoglobin when *A. aegypti* fed on naïve mice.

This negative effect was not seen in either *Anopheles* species. *An. stephensi* and *An. gambiae* feeding on naïve mice laid a mean of 97 $\pm$ 10.7 and 92 $\pm$ 8.21 fertile eggs respectively. When fed on mice sensitized to bites from *A. albopictus*, the mean number of fertile eggs viable for *An. stephensi* and *An. gambiae* was unchanged at 113 $\pm$ 8.2 and 91 $\pm$ 5.25 fertile eggs respectively.

Blood meal sizes were actually increased slightly when these mosquitoes fed on *A. albopictus* sensitized mice. When feeding on naïve mice, *An. stephensi* and *An. gambiae* blood meals contained a mean of 0.374 mg and 0.269 mg of hemoglobin respectively. When fed on sensitized mice *An. stephensi* and *An. gambiae* ingested a mean of 0.428 mg and 0.411mg of hemoglobin respectively (Figure2.22).

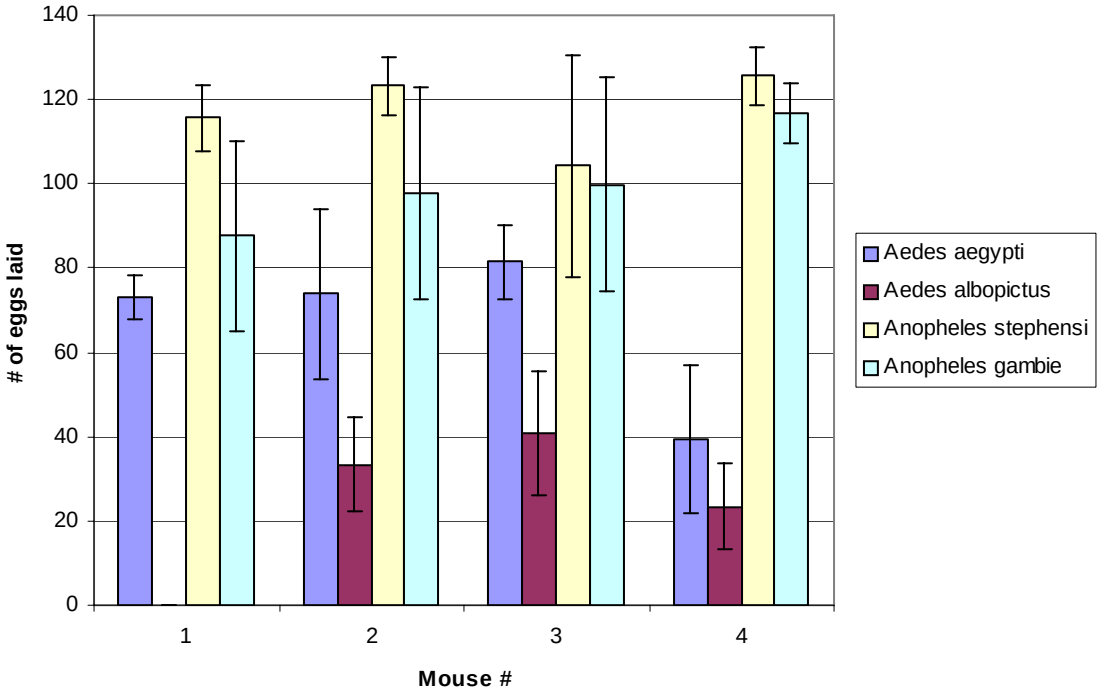

Figure 2.20: Effect of exposure to all 4 species used on number of eggs laid for mice sensitized to bites from *Aedes albopictus*. Error bars indicate ± one standard error (n=5 for each mouse/time point). *Aedes albopictus* were not able to feed on mouse one.

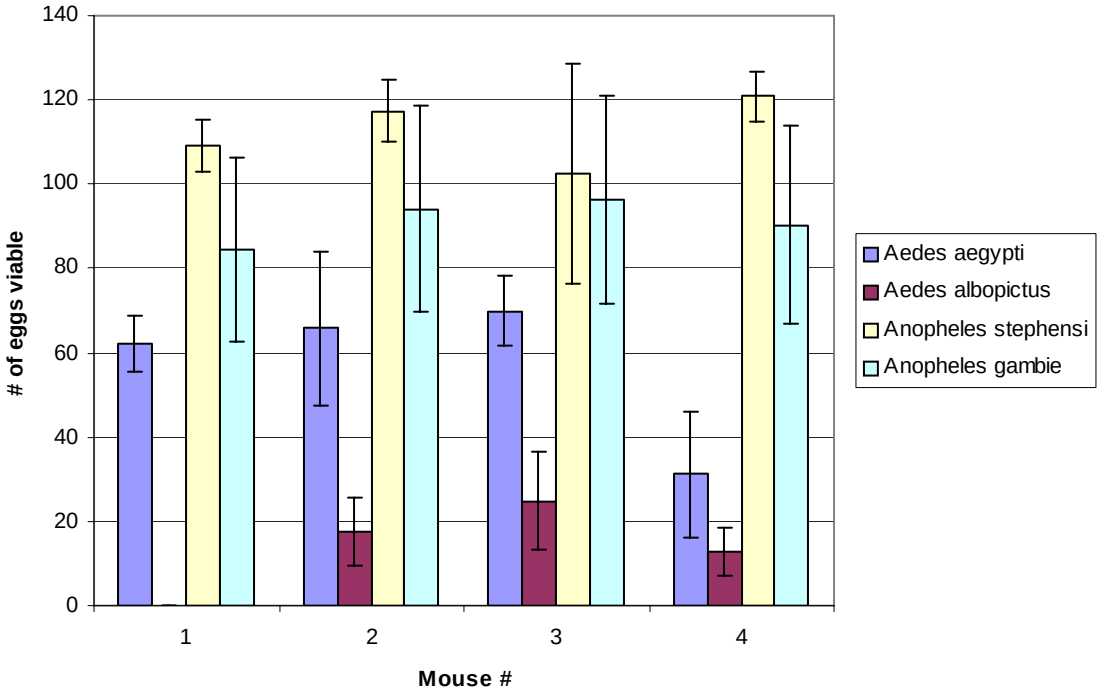

Figure 2.21: Effect of exposure to all 4 species used on number of eggs viable for mice sensitized to bites from *Aedes albopictus*. Error bars indicate ± one standard error (n=5 for each mouse/time point) *Aedes albopictus* were not able to feed on mouse one.

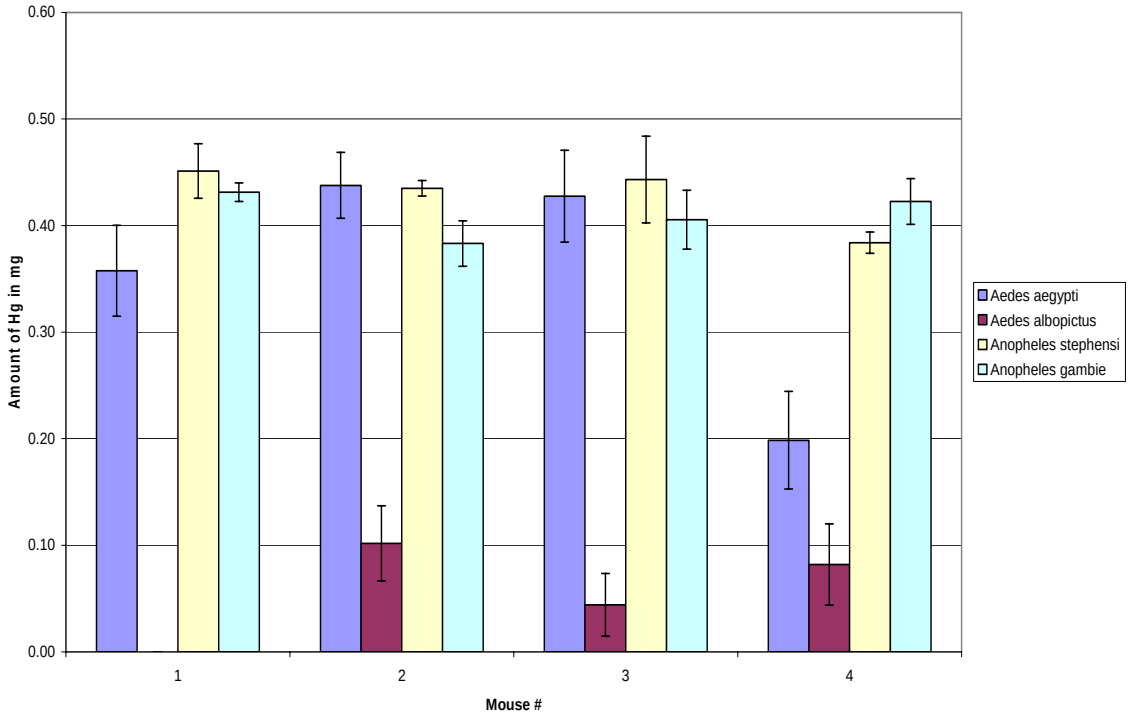

Figure 2.22: Effect of exposure to all 4 species used on blood meal volume for mice sensitized to bites from *Aedes albopictus*. Error bars indicate ± one standard error (n=5 for each mouse/time point) *Aedes albopictus* were not able to feed on mouse one.

When mice, sensitized to *An. stephensi* salivary antigens by four weekly exposures were used to feed *A. aegypti*, *A. albopictus*, and *An. gambiae* mosquitoes, only the latter species was significantly negatively affected. For *An. gambiae*, total egg production was significantly reduced to only 34 $\pm$ 8.1 eggs/female when compared to a mean of 99 $\pm$ 8.1 eggs/female when allowed to feed on naïve mice (Figure 2.23). Production of viable eggs was also reduced from a mean of 92 $\pm$ 8.2 viable eggs from naïve mice to 18 $\pm$ 8.2 eggs from the eggs produced from sensitized mice (Figure 2.24). The sensitized mice also had a negative effect on the ability of *An. gambiae* to obtain a blood meal. When fed on naïve mice, *An. gambiae* ingested an average of .269μg of hemoglobin (Figure 2.16). However, after feeding on the mice that had previously been exposed to *An. stephensi*, that average dropped by 46% to only .146μg hemoglobin (Figure 2.25). Conversely, being sensitized to An. stephensi bites had little to no effect on either *Aedes* species. *A. aegypti* averaged 88 $\pm$ 4.8 eggs/female when fed on naïve mice, compared to 98 $\pm$ 9 eggs when fed on the *An. stephensi* sensitized mice (Figure 2.23). *A. albopictus* had similar results, with a mean of 112 $\pm$ 1.7 eggs/female on naïve mice and a mean of 105 $\pm$ 8.1 eggs laid when allowed to feed on the *An. stephensi* sensitized mice (Figure 2.23).

Feeding on *An. stephensi* sensitized mice had no significant effect on the ability of either *Aedes* species to obtain a blood meal (Figure 2.25). On naïve mice, *A. aegypti* and *A. albopictus* ingested a mean of 0.307 mg and 0.407 mg of hemoglobin respectively. On week 5, after feeding on mice exposed to *An. stephensi*, *A. aegypti* and *A. albopictus* blood meals contained a mean of 0.342 mg and 0.320 mg of hemoglobin respectively.

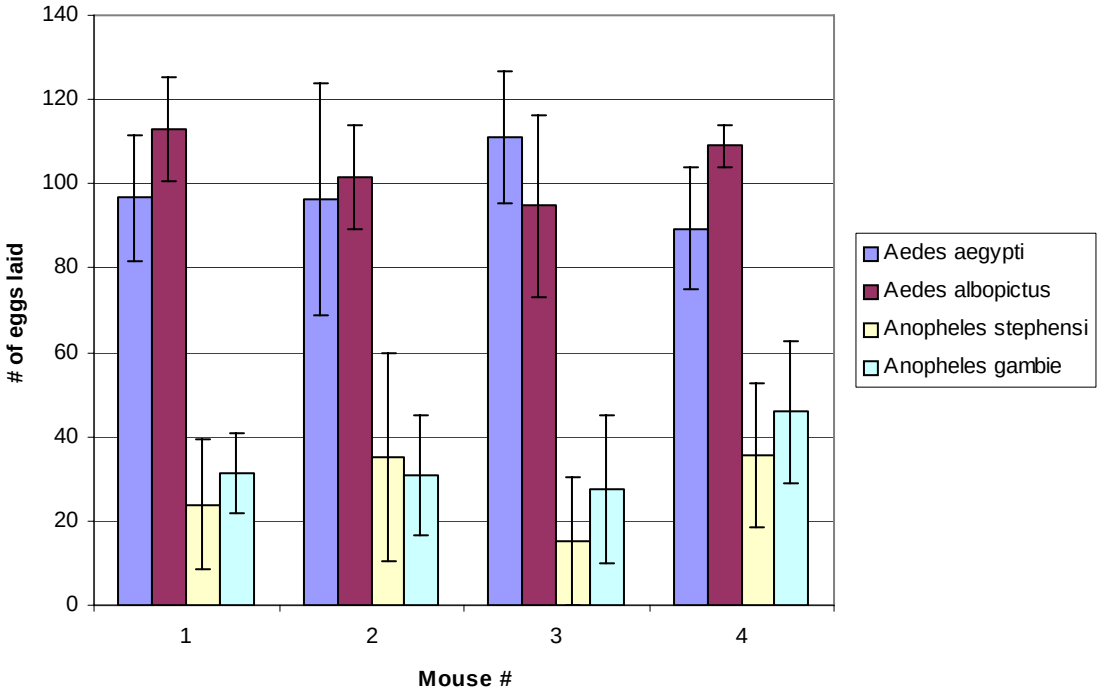

Figure 2.23: Effect of exposure to all 4 species used on number of eggs laid for mice sensitized to bites from *Anopheles stephensi*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

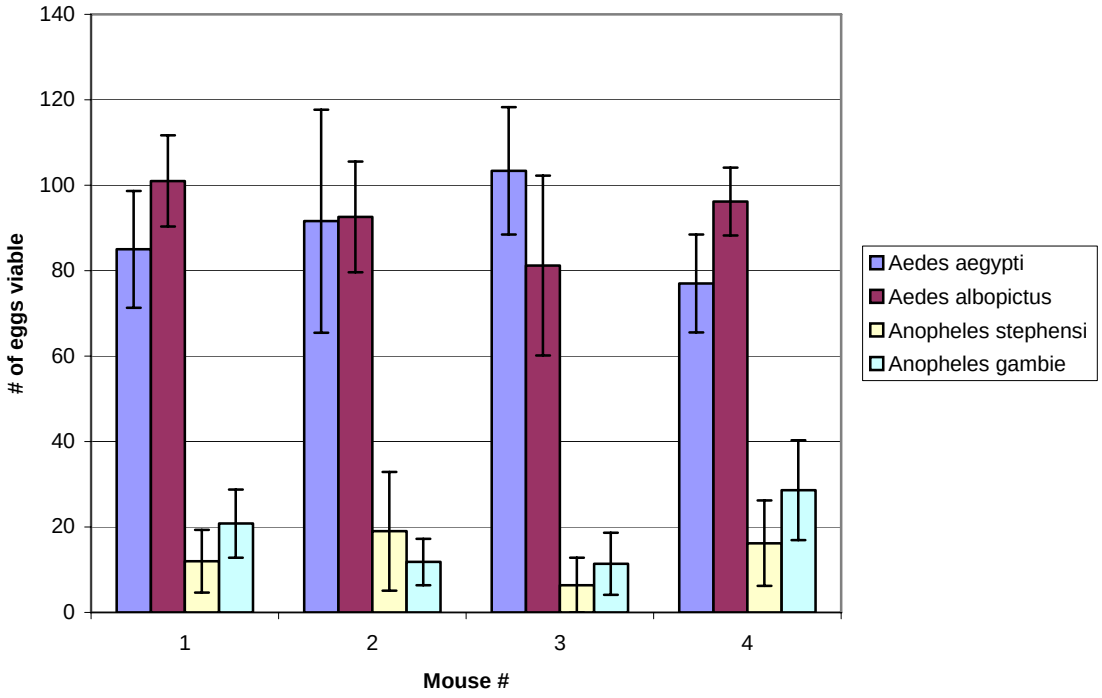

Figure 2.24: Effect of exposure to all 4 species used on number of eggs viable for mice sensitized to bites from *Anopheles stephensi*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

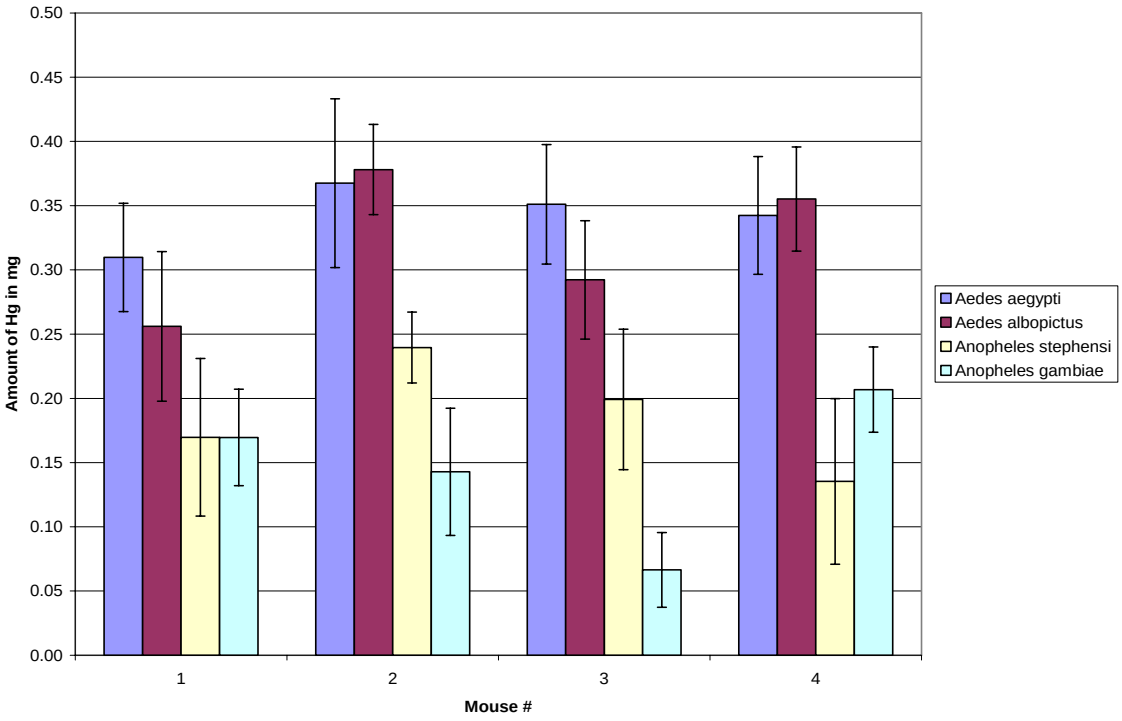

Figure 2.25: Effect of exposure to all 4 species used on blood meal volume for mice sensitized to bites from *Anopheles stephensi*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

Mice, sensitized to *Anopheles gambiae* salivary antigens by four weekly exposures, were used to feed *Aedes aegypti, Aedes albopictus*, and *Anopheles stephensi*. Only *An. stephensi* was adversely affected as total egg production was significantly reduced from a mean of 119 $\pm$ 3.8 eggs/female when blood fed on a naïve mouse to a mean of 68 $\pm$ 14.8 eggs when fed on a sensitized mouse (Figure 2.26). Production of viable eggs was also negatively affected. On naïve mice, *An. stephensi* averaged 97 $\pm$ 10.7 viable eggs (81.5% fertility rate), which is significantly higher than their week 5 average of 44 $\pm$ 13 eggs being viable (a fertility rate of 64.7%) (Figure 2.27). At least in part, the decrease in egg production was due to a negative effect on the ability of *An. gambiae* to obtain a blood meal. On week 1, *An. gambiae* blood meals contained an average of .269μg of hemoglobin. After feeding on the sensitized mice, this average dropped to .157μg hemoglobin, a 42% decrease in the volume of blood they were able to obtain (Figure 2.28).

The mice that were sensitized to bites from *An. gambiae* had little affect on the fecundity of the *Aedes* species. On week 1, *A. aegypti* and *A. albopictus* averaged 88 $\pm$ 4.8 and 112 $\pm$ 1.7 eggs laid respectively. When fed on the *An. gambiae* sensitized mice, their averages were 111 $\pm$ 8.7 and 108 $\pm$ 9.9 eggs respectively. The number of eggs that were viable was also not affected as *A. aegypti* and *A. albopictus* averaged 81 $\pm$ 3 and 108 $\pm$ 1.5 viable eggs on week 1 and 103 $\pm$ 9.3 and 100 $\pm$ 8.7 viable eggs on the *An. gambiae* sensitized mice (Figure 2.27). Feeding on *An. gambiae* sensitized mice had no significant effect on blood meals taken by either *Aedes* species. *A. aegypti* and *A. albopictus* ingested a mean of 0.307 mg and 0.407 mg of hemoglobin respectively when fed on week 1 (naïve) mice. When allowed to feed on the *An. gambiae* sensitized mice, blood meals were unchanged, averaging 0.381 mg and 0.383 mg of hemoglobin respectively (Figure 2.28).

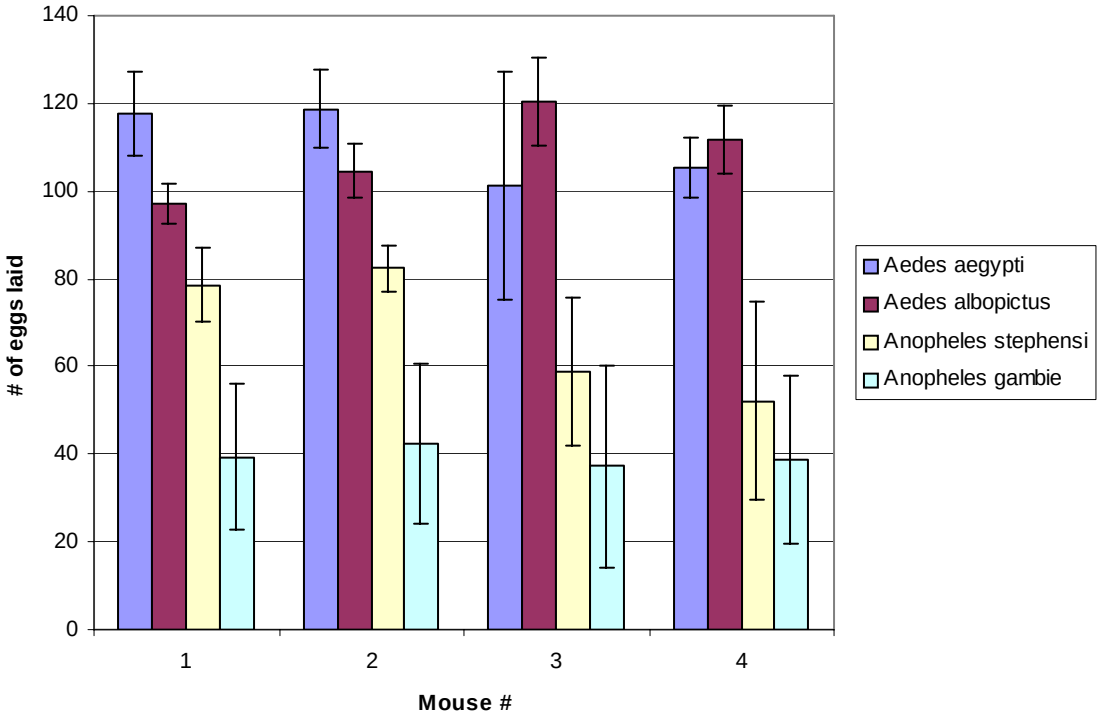

Figure 2.26: Effect of exposure to all 4 species used on number of eggs laid for mice sensitized to bites from *Anopheles gambiae*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

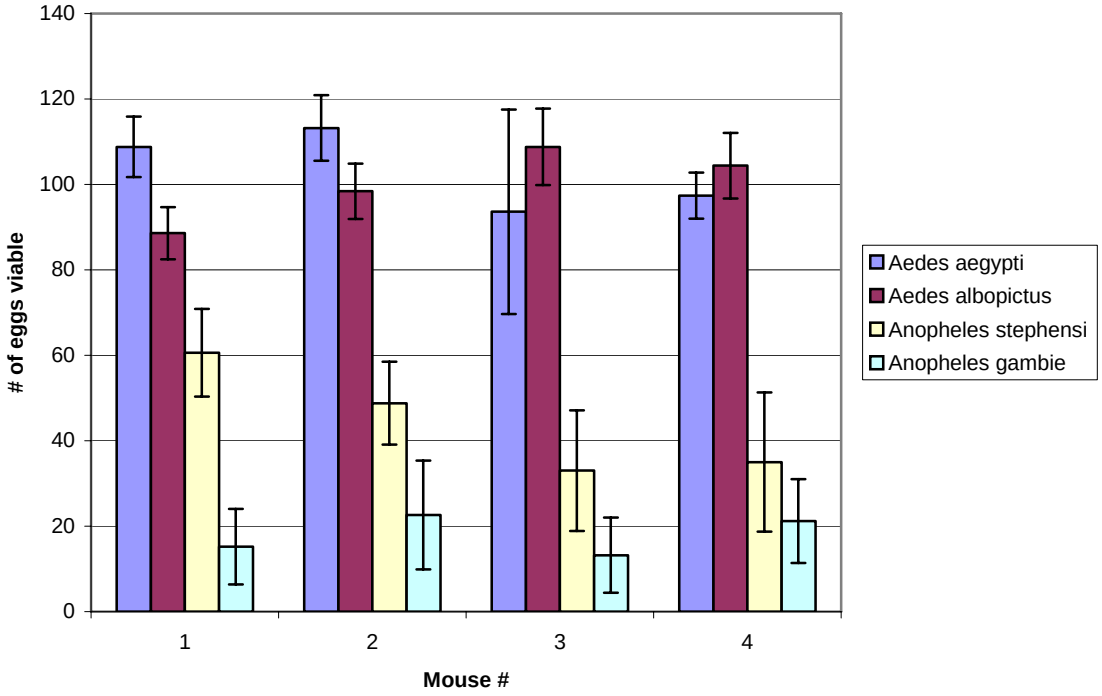

Figure 2.27: Effect of exposure to all 4 species used on number of eggs viable for mice sensitized to bites from *Anopheles gambiae*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

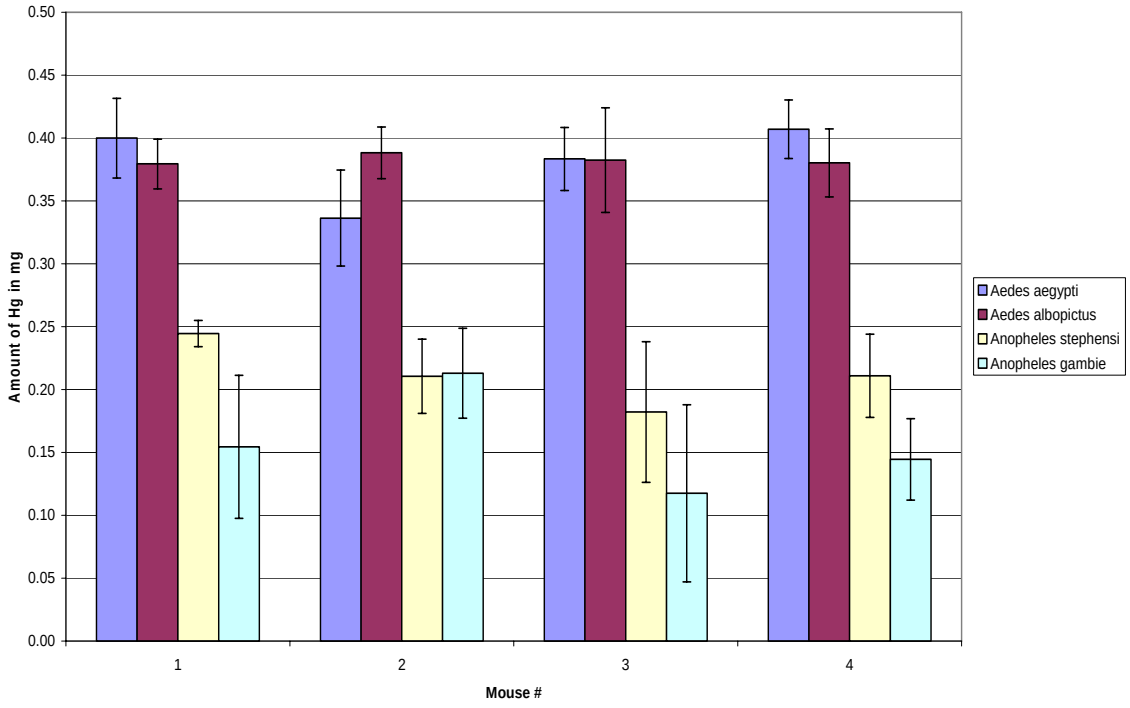

Figure 2.28: Effect of exposure to all 4 species used on blood meal volume for mice sensitized to bites from *Anopheles gambiae*. Error bars indicate ± one standard error (n=5 for each mouse/time point).

Western Blot Analysis

Western blot analysis provided evidence of seroconversion in mice exposed to biting by all four species of mosquitoes used in this study. This approach was also used to examine cross-reactivity of salivary antigens between the four mosquito species.

Aedes aegypti sensitized mice

Serum from the mice sensitized to bites from *A. aegypti* for five weeks yielded 6 different bands in the *A. aegypti* salivary gland extracts (SGE). The largest band seen was of undetermined size as it was above the highest marker in the size ladder used. The remaining 5 bands were approximately 203, 100, 37, and 30 kDa respectively (Figure 2.29). None of the remaining 3 species yielded any detectable bands.

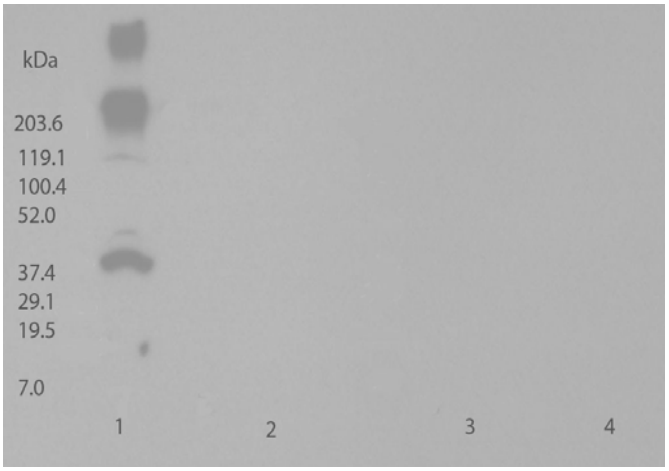

Figure 2.29: Western results for mice sensitized to bites from *Aedes aegypti*, 5 min. exposure (lane 1=*Aedes aegypti*, 2=*Aedes albopictus*, 3=*Anopheles stephensi*, 4=*Anopheles gambiae*)

Aedes albopictus sensitized mice

Serum from the mice sensitized to bites from *A. albopictus* bound to three proteins in the *A. albopictus* SGE, and also showed evidence for some cross reactivity with 2 of the other 3 species of mosquitoes. In reaction to the *A. albopictus* SGE, bands were detected at approximately 60, 30, and 28 kDa. In response to the *A. aegypti* SGE, there were 3 bands produced of approximately 60, 30, and 10 kDa respectively. In response to the *An. gambiae* SGE, a single band was detected that was approximately 15 kDa in size. No bands were detected in response to *An. stephensi* SGE (Figure 2.30).

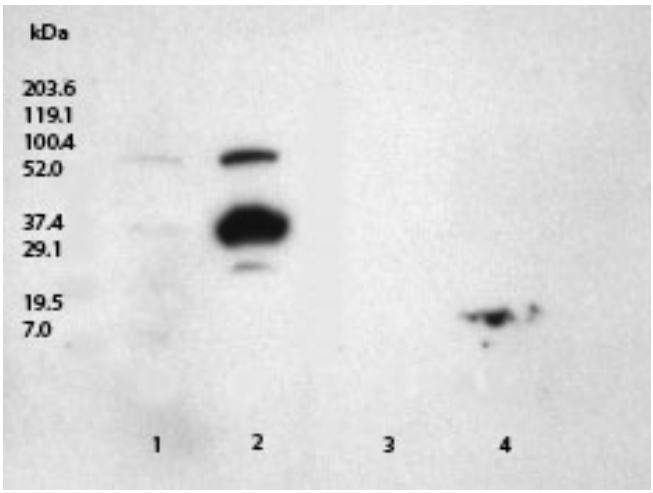

Figure 2.30: Western results for mice sensitized to bites from *Aedes albopictus*, 2 min. exposure (1=*Aedes aegypti*, 2=*Aedes albopictus*, 3=*Anopheles stephensi*, 4=*Anopheles gambiae*)

Anopheles stephensi sensitized mice

The serum collected from mice sensitized to *An. stephensi* yielded 3 bands with approximate sizes of 203, 52, and 20 kDa respectively when tested against *An. stephensi* SGE. This serum also reacted with *An. gambiae* and *A. albopictus* SGE as 1 band was detected from each that was approximately 60 and 32 kDa in size respectively. The 32 kDa band appears to correspond with most immunoreactive band seen when *A. albopictus* SGE was probed with serum from an *A. albopictus*-sensitized mouse. Similarly, the 60 kDa band in *An. gambiae* SGE corresponds with the most immunoreactive band seen with serum from *An. gambiae*-sensitized mice. *A. aegypti* SGE did not react with the serum (Figure 2.31).

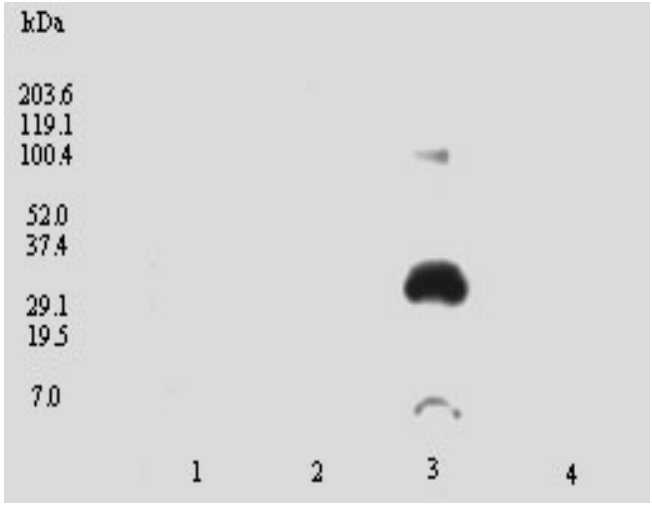

Figure 2.31: Western results for mice sensitized to bites from *Anopheles stephensi*, 1 min. exposure (1=*Aedes aegypti*, 2=*Aedes albopictus*, 3=*Anopheles stephensi*, 4=*Anopheles gambiae*)

Anopheles gambiae sensitized mice

Serum harvested from the mice sensitized to bites from *An. gambiae* yielded 1 band in response to *An. gambiae* SGE that was approximately 52 kDa in size. *A. albopictus* SGE also showed a reaction with this serum as one band was produced that was approximately 32 kDa in size. Again, this corresponded to the most immunoreactive band seen with serum from *A. albopictus*-sensitized mice. Neither of the other 2 species used in this study yielded any detectable bands (Figure 2.32).

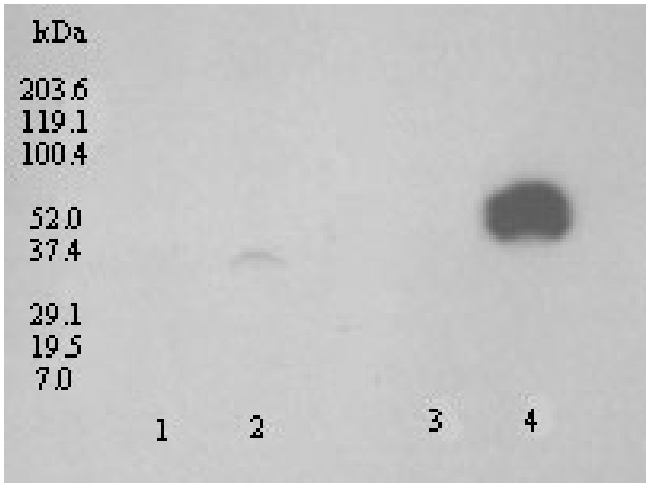

Figure 2.32: Western results for mice sensitized to bites from *Anopheles gambiae*, 10 min. exposure (1=*Aedes aegypti*, 2=*Aedes albopictus*, 3=*Anopheles stephensi*, 4=*Anopheles gambiae*)

Effect of Repeated Host Exposure to Mosquito Feeding on Egg Production and Viability in B-cell Knockout Mice and Controls

I tested the hypothesis that the negative effects on mosquito performance found above are dependent on host seroconversion, by repeating the experiment using B-cell knockout mice that are unable to produce antibodies, along with strain-specific (B6) controls. Only *Aedes aegypti* was used for this portion of the experiment (they are the easiest to rear/maintain out of the 4 species used in this experiment). When B-cell knockout mice were exposed to five consecutive weekly bouts of blood feeding by mosquitoes, no decrease in blood meal size, egg production, or in egg viability was observed. In the B6 control mice, there was a progressive decrease in the size of the blood meal taken, in the number of eggs laid, and in the viability of those eggs (i.e. in the proportion hatching).

B-cell Knock Out Mice Sensitized to *Aedes aegypti*

When *Aedes aegypti* fed on naïve B-cell KO mice, they produced a mean of 117 $\pm$ 5.2 eggs. Unlike the other mice, there was no statistically significant, observable trend of decreasing egg production. On week 5, the number had actually increased to 124 $\pm$ 3 eggs, although this was not significant (p=.057) (Figure 2.36). The same can be said for the number of eggs that were viable. Week 1 began with a mean of 113 $\pm$ 4.4 larvae produced. Again, there was no statistically significant, observable trend seen in the number of eggs being viable. Week 5 also showed an increase with the number of larvae produced and ended with a mean of 120 + 2.4 larvae produced (p=.024) (Figure 2.37). Blood meal sizes also were not affected, as week 1 blood meals contained had an average of 0.443 mg hemoglobin. By week 5, the amount of

hemoglobin ingested was unchanged, with the mean being 0.439 mg hemoglobin (p=.727) (Figure 2.38).

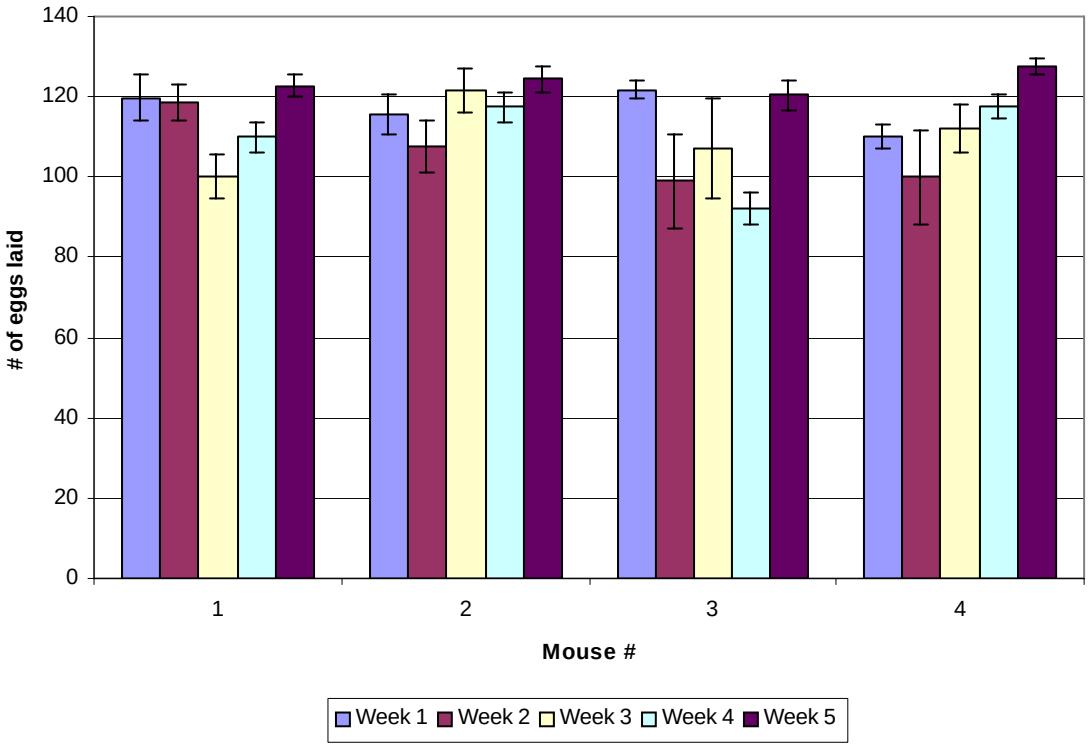

Figure 2.33: Effect of exposure to *Aedes aegypti* feeding on the number of eggs laid per blood meal for B-cell KO mice. Error bars indicate ± one standard error (n=10 for each mouse/time point).

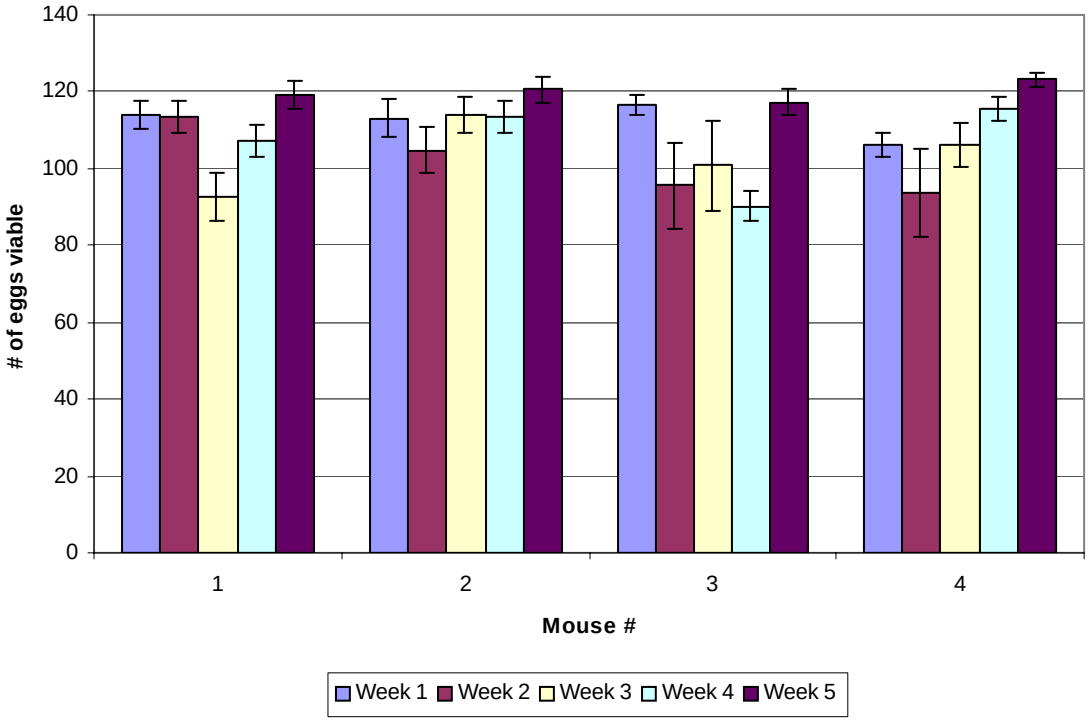

Figure 2.34: Effect of exposure to *Aedes aegypti* feeding on the number of eggs viable per blood meal for B-cell KO mice. Error bars indicate ± one standard error (n=10 for each mouse/time point).

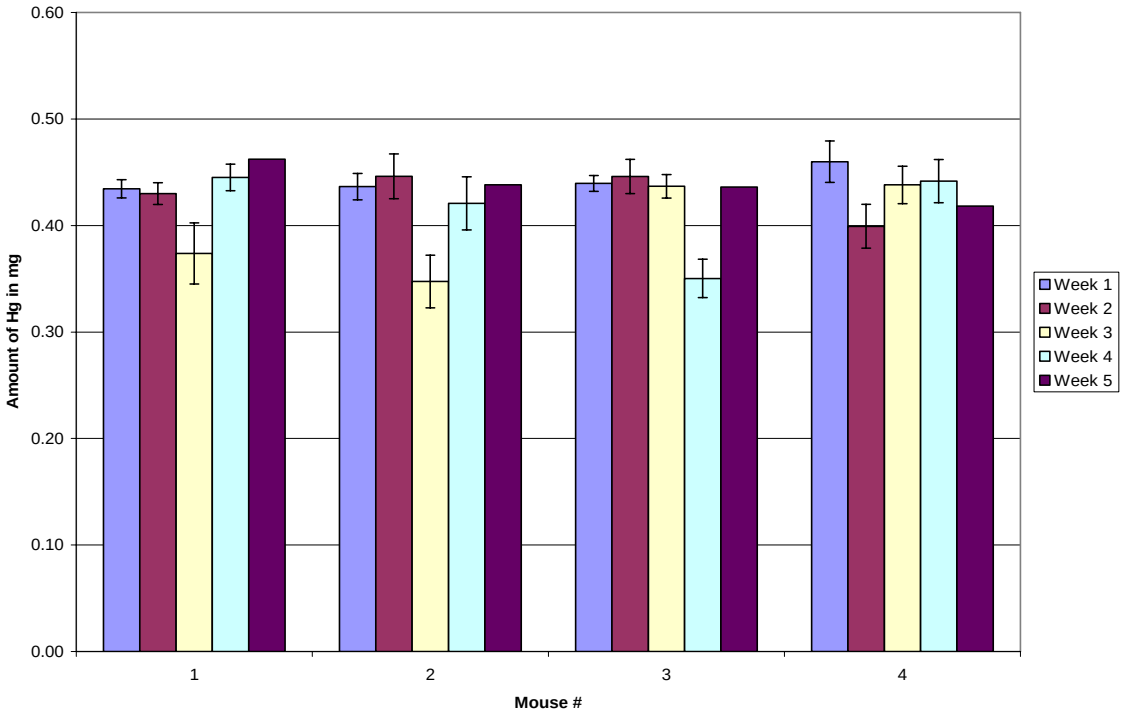

Figure 2.35: Effect of exposure to *Aedes aegypti* feeding on blood meal size for B-cell KO mice. Error bars indicate ± one standard error (n=10 for each mouse/time point).

B-cell KO Control Mice Sensitized to *Aedes aegypti*

When *Aedes aegypti* fed on the naïve control mice, they laid a mean of 111 ± 6.6 eggs. The mean number of eggs continued to decrease with each consecutive week, but this was not significant until week 3 when the mean dropped to 82 ± 7.9 eggs ($p=.001$). The trend continued for weeks 4 and 5 as the mean dropped to 83 ± 4.3 and 62 ± 18.8 respectively ($p=.002$, $p<0.001$ respectively) (Figure 2.33). A similar trend of weekly decreases was also seen in the production of viable eggs. Week 1 began with a mean of 108 ± 6.7 eggs. In subsequent weeks, viable egg production declined significantly such that by weeks 4 and 5, the mean had dropped to 74 ± 4.5 and 49 ± 16.2 larvae respectively ($p<0.001$, $p<0.001$). This decline was seen in all 4 mice (Figure 2.34).

The decline in numbers of viable eggs was not only due to a decrease in the total number of eggs produced, but also to a progressive decline in egg viability. On week 1, 97% of the eggs were viable. On week 5, this had declined to a 79% viability rate.

Blood meal sizes, measured in milligrams of hemoglobin ingested, were also negatively affected over the 5-week period. Week 1 showed a mean of 0.430 mg hemoglobin. Statistically significant decreases were seen with each progressive week until week 5, which had a mean of 0.180 mg ($p<0.001$) (Figure 2.35).

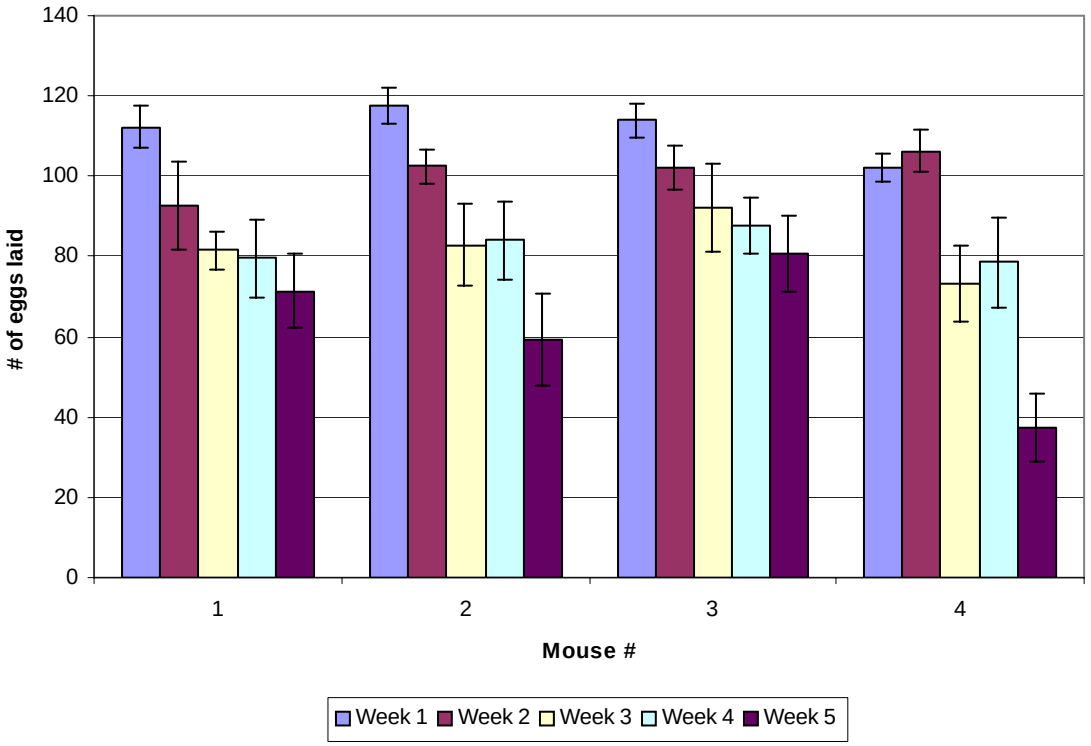

Figure 2.36: Effect of exposure to *Aedes aegypti* feeding on the number of eggs laid per blood meal for B-cell control mice. Error bars indicate ± one standard error (n=10 for each mouse/time point).

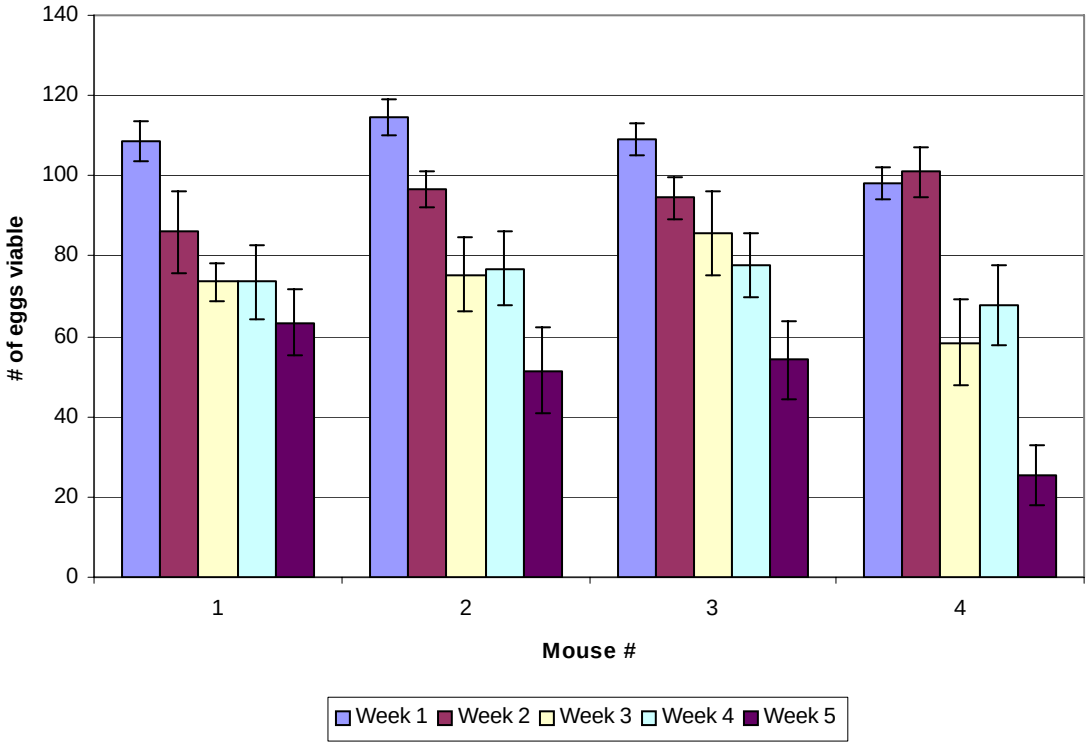

Figure 2.37: Effect of exposure to *Aedes aegypti* feeding on the number of eggs viable per blood meal for B-cell control mice. Error bars indicate ± one standard error (n=10 for each mouse/time point).

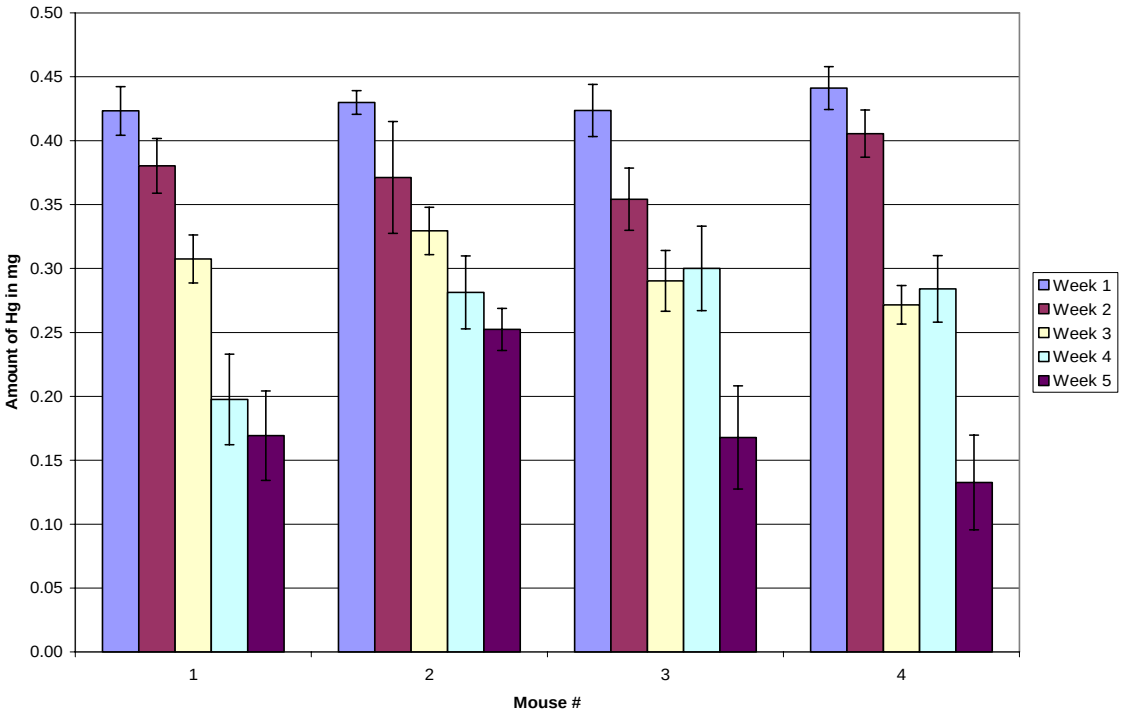

Figure 2.38: Effect of exposure to *Aedes aegypti* feeding on blood meal size for B-cell control mice. Error bars indicate ± one standard error (n=10 for each mouse/time point).

Western Blot Analysis of B-cell KO and Control Mice

Control Mice Sensitized to *Aedes aegypti*

The serum harvested from the control mice only reacted with the *A. aegypti* SGE and produced only one band with an approximate size of 70 kDa (Figure 2.39). This band does not correspond to any of the five bands bound by serum from *A. aegypti*-sensitized BALB/c mice. The reason for the differences between this blot and that seen with serum from BALB/c mice are not clear, although they may relate to differences between strains. Nevertheless, this blot does establish that the B6 control mice have seroconverted by week five.

B-cell KO Mice Sensitized to *Aedes aegypti*

The absence of seroconversion in the B-cell knockout mice was confirmed, as there were no bands detected in the western blot (Figure 2.39).

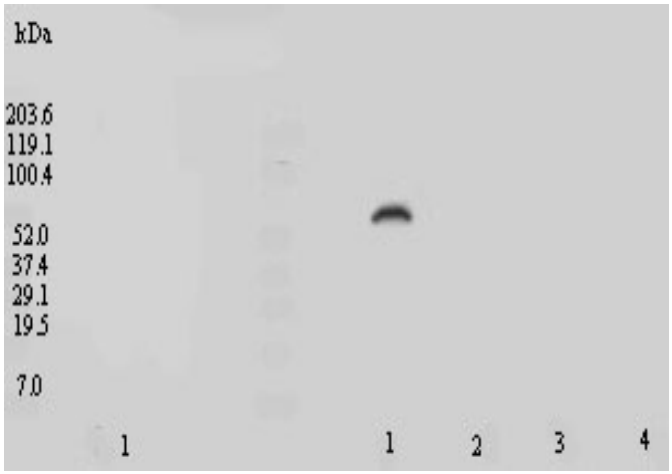

Figure 2.39: Western results for B-cell KO and control mice, 1 min. exposure (1=*Aedes aegypti*, 2=*Aedes albopictus*, 3=*Anopheles stephensi*, 4=*Anopheles gambiae*) The different number of bands seen here when compared to the western for the mice sensitized to A. aegypti sensitized mice used in the previous portion of the experiment may be due to the length of the exposure.

Discussion

Vertebrate hosts defend against blood loss with a complex set of responses including platelet aggregation, vasoconstriction, and coagulation (clotting), collectively called hemostatic responses (3). Mosquito salivary components play a critical role in blood feeding, by inhibiting each of these components of hemostasis (2, 3, 12, 47, 59). In order to carry out these functions, salivary components are injected into the skin of the vertebrate host, and it is well known that vertebrate hosts exposed to mosquito feeding will respond by producing antibodies against antigenic components of the saliva, a process termed seroconversion (1). This led us to the hypothesis that host seroconversion would impact the ability of the mosquito to feed efficiently. As female mosquitoes use the blood meal primarily to produce eggs, it is likely that any decrease in feeding efficiency would lead to a reduction in fitness.

To test this hypothesis we exposed mice to mosquito biting weekly for five weeks, and measured the amount of blood retained by the mosquitoes (which correlates strongly with egg production) (5, 63), and the total number of eggs and the number of viable eggs produced per mosquito as a direct measure of fitness. The experiment was repeated with two *Aedes* species, *A. aegypti* and *A. albopictus*, and two *Anopheles* species, *An. stephensi* and *An. gambiae*, to ensure that our conclusions apply generally to mosquito-host interactions. All four mosquito species used in this study laid more eggs and acquired larger blood meals from the naïve BALB/c mice as opposed to the sensitized mice (weeks 3-5). From the third week on, a progressive decline was seen in the number of eggs produced following each blood meal. An even more pronounced progressive decline in the percentage of eggs that were viable was observed. Furthermore, there was a decrease in the volume of blood the mosquitoes were able to obtain during the fourth and fifth weeks. Given that these effects were seen in all four-mosquito species, these data suggest

that this consequence of prior exposure to mosquito biting is a general aspect of the mosquito/host interaction and this is not just a special case for one particular mosquito species.

The progressive decline seen in egg production, egg viability, and blood meal size is dependent on the host seroconverting. Western blot analysis indicated that all mice had seroconverted by the end of the five-week period, with antibody binding to between one and six salivary components for each mosquito species. To test the hypothesis that this seroconversion is necessary for the fitness-reducing effect of exposure to mosquito feeding, we repeated the experiment using B-cell knock out mice that cannot produce antibodies. This failure to seroconvert was confirmed when the western blot using the mouse serum from one of these mice yielded no bands. No decrease in egg production, egg viability, or blood meal size was seen during the course of five weekly exposures to *A. aegypti* feeding. When mosquitoes fed on control B6 mice with the same genetic background as the B-cell knockouts, the progressive decline in blood meal size and egg production was again observed. This finding leads us to accept our hypothesis that host seroconversion does impact the ability of the mosquito to feed efficiently, leading to a reduction in reproductive potential. This experiment also demonstrates that the effect is not specific to one mouse strain, as it was observed in both BALB/c and black 6 mice, and also that it occurs despite the fact that different salivary proteins were recognized by the two mouse strains. Further, it demonstrates that the effects are not simply a consequence of the aging of the mice (e.g. young mice are a better food resource that older mice).

Some cross reactivity was observed between mosquitoes in the same genus, but not between genera. For example, mice sensitized to bites from *A. aegypti* for five consecutive weeks also seemed to have a negative affect on *A. albopictus*. The number of eggs produced, fertile eggs, and the volume of their blood meals were significantly reduced, compared to when

A. albopictus fed on naïve mice. On the other hand, meal size and egg production by *An. stephensi* and *An. gambiae* were not reduced. When mice were sensitized to one of the *Anopheles* species, the other *Anopheles* species was impacted but there was no effect on either *Aedes* species. These effects are likely due to the presence of cross-reacting antigenic proteins present in saliva of the mosquito. It is likely that there is enough similarity to allow cross-reaction in closely related mosquitoes, but not in more distantly related species. For example, the antihemostatic enzyme apyrase has 83% sequence identity at the amino acid level when *A. aegypti* is compared to *A. albopictus*, but there is only 62% identity when *A. aegypti* is compared to *An. stephensi*, and 49% identity when compare to *An. gambiae* apyrase.

This conclusion was supported in part by the western blot analysis. Certainly, mice strongly seroconverted in response to biting from all four mosquito species tested. Evidence for antigenic cross-reactivity as an explanation for the cross-sensitization effects seen is less clear-cut. For example, serum from *A. albopictus* sensitized mice interacted with several proteins in *A. aegypti* SGE, which could explain the adverse effects observed when *A. aegypti* fed on *A. albopictus*-sensitized mice. However, this serum also bound to a single band in *An. gambiae* SGE, but no fitness impact indicating cross-sensitization between these species was observed, which suggests that some antigenic proteins may be more important than others in producing an adverse effect..

A similar argument applies to recognition of a 35 kDa band in *A. albopictus* SGE by serum from mice sensitized to either *Anopheles* species. The *A. aegypti* western indicated no cross-reactivity between species, but the data shows that *A. albopictus* was significantly negatively affected. We speculate that cross-reactivity was not observed in the western blot because serum dilutions were optimized to reveal reaction with *A. aegypti* antigens, and these conditions were not sensitive

enough to reveal cross-reaction with *A. albopictus* antigens. This could be repeated to confirm this theory.

Our findings have important ecological implications. Mosquito reproductive potential might be dependent on the immunological status of the host population. For example, reproductive potential would be higher if the hosts' population included many young, immunologically naïve individuals, compared to feeding on a population of older, seroconverted hosts. Additionally, in nature, hosts could be exposed to multiple mosquito species. It follows that the antibody response from the host, and the consequent fitness impact on mosquitoes, would be dependent on the intensity of exposure to both conspecific and taxonomically related mosquitoes.

There are two possible mechanisms to explain our observations. The first possibility is that the antibodies produced by the host in response to the mosquito saliva bind to and block the activity of the antihemostatic enzymes. If this is what is happening, we would predict that the ability of the mosquitoes to inhibit hemostasis would be impaired, leading to a longer time to obtain a normal size blood meal, or to smaller blood meals. The second possibility is that there is some kind of antibody-dependent post-ingestive process producing damage in the gut. It is important to note that the mosquito reingests over 25% of the saliva secreted during the meal (61). This implies that antigens, antibodies, serum proteins including compliment, and immune cells are all present in the blood meal in addition to the hosts' blood. One candidate for a post-ingestive mechanism is activation of the compliment system by antigen-antibody complexes. This system is a very complex enzyme cascade system consisting of numerous serum glycoproteins. The system has two distinct pathways, but both ultimately end in the production of the membrane attack complex (MAC). The MAC, once formed, is responsible for creating a

transmembrane pore that leads to lysis of the cell (35). Activation of complement in the gut therefore is likely to lead to damage to the gut epithelium. We are currently conducting experiments with complement (C3) knockout mice to test this hypothesis. However, there are other possibilities, including activation of lymphocytes in the blood meal by antibody-antigen complexes, leading to secretion of toxic molecules such as tumor necrosis factor or nitric oxide.

Analysis of the data suggests that both possibilities may be happening. Over the course of the experiment, blood meal sizes do decline, but not significantly until the last two weeks. This observation is consistent with the first possibility that antibodies bind to and block the activity of antihemostatic enzymes. In this regard, it is interesting to note that serum from *An. stephensi* sensitized mice bound to and inhibited apyrase activity (32), but this did not in itself reduce the ability of mosquitoes to feed, measured as probing time prior to ingestion of blood. However, apyrase acts mainly to remove ADP from the injured intravascular tissue produced during probing, prior to penetration of the blood vessel. Antibody titers could be low in the intravascular tissue, so by the time apyrase is exposed to high anti-apyrase titers in the blood this enzyme would have already completed its pharmacological function, with the result that little effect on feeding is seen.

We observed that the numbers of eggs produced begin to decline earlier than do the blood meal sizes. This observation suggests that for some blood meals, fewer eggs are being produced but a normal amount of blood is being taken in. This implies that there is an increased cost in processing the meal, so there is less blood available to produce eggs. This observation favors the hypothesis that some kind of antibody-dependent post-ingestive process is going on in the gut of the mosquito.

My findings are somewhat surprising, in that it seems counter-intuitive (given the commonly observed abundance of mosquitoes) that mosquito reproductive success could be so dramatically reduced in the field. It is possible that the effects I observed are dependent on the specific antibody isotype produced by the host. Prolonged exposure to antigens is known to result in antibody isotype switching, which accounts for the changing skin responses to mosquito bites described earlier. It remains to be determined if such prolonged exposure would cause mice to switch to an antibody isotype that does not produce the fitness-reducing effects. For this reason, I suggest that these experiments be repeated for a longer time frame, and include identification of the isotypes (IgE, IgM, IgG) produced as well as their fitness effects at different points in the process.

My results demonstrate that host seroconversion has a negative effect on the reproductive potential of mosquitoes. Differences in reproductive potential can lead to differential fitness if there are differences between genotypes in reproductive potential. Salivary proteins are highly variable; for example, in an unpublished study I found 50 different alleles for the short D7 in a sample of 115 sequences derived from eight different *A. aegypti* populations. If these allelic differences result in differential recognition by host antibodies, it is likely that individual mosquitoes that possess alleles that escape immune recognition will enjoy a relative fitness advantage. In essence, hosts that are exposed to mosquito attacks will generate antibodies, and on average, these antibodies will recognize common alleles in the mosquito population. Mosquitoes that possess antigenically novel alleles, produced by mutation, recombination, or by immigration from other populations, will have a fitness advantage. However, as those alleles become more common, a larger portion of the host population will have been exposed to these novel alleles and they will gradually become less advantageous. This process should favor the

accumulation of a high amount of antigenic variability in salivary gland proteins. Examination of this hypothesis is likely to be a productive area of future research.

My findings have practical applications for researchers maintaining mosquito colonies. It would be in the researchers best interest to avoid using the same rodents for extended periods of time as this could ultimately adversely impact mosquito colony performance once the rodents had seroconverted. More significantly, our data suggests that it may be possible to exploit the fitness reducing effect of seroconversion to reduce mosquito populations, for example, vaccination of host populations could improve the fitness-reducing effects by manipulating the specific antigens that are recognized, or the antibody isotypes that are produced (so as to maximize the post-ingestive effects). Our findings may also have some implications for disease transmission. Many pathogens are delivered into the intravascular tissue of the skin during the probing phase of the blood meal. Any biological effect that reduces the efficiency of feeding has a possibility of increasing disease transmission by increasing the amount of probing necessary to obtain a blood meal. For example, *Plasmodium gallinaceum* reduces the titer of apyrase in the saliva (31). The consequence of this is that *A. aegypti* mosquitoes are unable to fully inhibit platelet aggregation. This results in the meal being frequently interrupted and the mosquito must repeatedly probe to reacquire a blood vessel with the result that more sporozoites are injected into the host (33). If host seroconversion impedes the feeding process, it could similarly have the effect of enhancing pathogen transmission. On the other hand, activation of toxic immune responses in the blood meal could indirectly increase parasite mortality in the gut, reducing the probability of horizontal transmission of pathogens from an infected host to the mosquito. Further study will be required to help distinguish between these two possibilities.

In conclusion, this experiment has shown that host seroconversion results in a fitness reduction for mosquitoes. More specifically, it decreases the numbers of eggs they can produce, the proportion of those eggs that are viable, and the volume of the blood they ingest. These findings have important ecological and evolutionary applications as well as applications for researchers maintaining colonies of mosquitoes and studying disease transmission.

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