Exploratory Study of Ectoparasites of Medical & Veterinary Importance in Alabama

by

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Abstract

Ectoparasites play a critical role in affecting the health and economic prosperity of both human and animal populations. This thesis presents a study focusing on two prominent ectoparasites within Auburn, Alabama: horn flies (Diptera: Muscidae: *Haematobia irritans irritans*) and mosquitoes (Diptera: Culicidae: *Culex erraticus*). The objective of this study was to elucidate the efficacy of a novel vaccine for cattle against *Haematobia irritans irritans* and to determine the viral profile within *Culex erraticus* found within the Auburn Fisheries.

This thesis encompasses a comprehensive literature review, field collections, RNA extractions, Next Generation Sequencing, and live animal investigations. The goals were to determine the physiological effects vaccinated cattle play in feeding behavior of *Haematobia irritans irritans* and gather information on the viral profile of *Culex erraticus* found in the Auburn Fisheries. Both horn flies and mosquitoes are ectoparasites that can have major economic consequences in the livestock industry, but also play a negative role in a medical (human and veterinary) context by spreading disease.

Findings revealed vaccinating heifers against thrombostasins altered horn fly feeding based upon smaller blood meal volumes in the midguts of horn flies that fed on vaccinated animals versus ones that fed on control animals. This is done by measuring the blood volume of horn fly mid guts after a 12 hour starve followed by a 25-minute feeding challenge. Consistent with this finding, horn fly mortality was higher in flies feeding on vaccinated heifers during a 3-day cage challenge compared to flies feeding on control animals during the same challenge. This suggests vaccination induced a negative energy balance in flies that resulted in a shorter life spans. The viral profile of *Culex erraticus* has yet to be determined, but high quality Culex RNA

samples have been successfully sequenced and the resulting sequence data set is currently undergoing bioinformatic analysis.

The knowledge gained from this research provides important support for the commercialization of the vaccine which would provide novel tools for cattle producers to use against horn flies. Furthermore, full sequencing of the *Culex erraticus* will result in characterization of viruses found in mosquitoes which is vital information needed to develop sustainable management practices for mitigating the negative effects of these ectoparasites on both human and animal populations. By generating this knowledge, we will be better able to manage and protect not only our livestock, but ourselves as well.

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TS	Thrombostasins
CDC	Centers for Disease Control and Prevention
WNV	West Nile Virus
EEE	Eastern Equine Encephalitis
NGS	Next Generation Sequencing

Chapter 1

Literature Review

1.1.1 Introduction

The motivation for this research is to first educate and protect ourselves from the escalating threat of vector borne zoonotic disease due to agricultural expansion. Another motivator is to validate more efficient ways to increase cattle production to reduce financial loss, combat global food insecurity, and decrease insecticide resistance. This will be accomplished by first investigating the viral profile within *Culex erraticus,* a common mosquito found around the Auburn fisheries; as well as determining the efficacy of a novel recombinant protein vaccine by comparing the physiological effects of *Haematobia irritans irritans,* horn flies, feeding off vaccinated or unvaccinated cattle.

According to the Food and Agriculture Organization of the United Nations, global food security is a rising problem as 9.2% of the global population faced hunger in 2022. This number has risen since 2019 when hunger was faced by 7.9% of the world's population (FAO et al. 2023). Food security is greatly impacted by the growing global population, but also challenged by climate change, which can make crops more difficult to grow and animals more difficult to raise (Ericksen et al. 2009).

The ever-growing need for food and shelter has led to the expansion of human societies into previously wild territories and has promoted the use of more insecticides. These actions often lead to an abundance of novel issues which include unsustainable farming habits, habitat loss, increased contact between humans and wildlife, which in turn leads to a higher risk of novel

and re-emerging disease transmission (Barbier 2021), and insecticide resistance. All of which ultimately negatively impact human health and are exacerbated by climate change and globalization.

1.1.2 Introduction to Vector Borne Diseases

Zoonotic diseases are illnesses caused by pathogens that make the jump between animals and humans. They are caused by harmful microbes including fungi, bacteria, viruses, and eukaryotic parasites (CDC 2021) Zoonotic diseases are not only transmitted through direct contact with infected animals, their bodily fluids, or their environment, but also through indirect contact such as consumption of contaminated food, water, or vector bites, such as mosquitoes (CDC 2021). Many of these diseases have animal hosts, which are known as reservoirs. These animals are where the pathogens naturally reside without serious symptoms of illness. However, occasionally there are spill over events between these animals to humans, which may cause serious and deadly diseases (Ellwanger and Chies 2021).

As stated by the Centers for Disease Control and Prevention (CDC 2019), scientists have estimated that out of every 10 infectious diseases found in people, more than 6 can be spread zoonotically and out of every 4 emerging infectious diseases, 3 are of zoonotic origins. According to the World Health Organization, the risk of zoonotic diseases occurring increases along with urbanization and destruction of natural habitats due to the increased contact between humans and wild animals (WHO 2020).

A recent example of a deadly zoonotic disease in an agricultural context would be the virus responsible for the 2009 Swine flu outbreak, H1N1 (Dotis and Roilides 2009). The disease originated in swine and jumped into people causing an outbreak similar to the influenza in

Mexico mid-February 2009 (Al Hajjar and McIntosh 2010). Due to airline travel and globalization, the virus spread at unprecedented rates and became a global pandemic of the highest degree by June of 2009 (Mena et al. 2016). The likelihood of pathogens jumping from animal to human is more likely to occur when people come in contact with animals. This can happen when they encounter new environments and/or can also happen when they surround themselves with animals in high numbers (as in agriculture). Interactions as such are bound to increase when humans encroach further into wild territories due to urbanization for shelter, food, and agriculture. This is a growing concern as food insecurity continues to rise (Baquedano et al. 2021).

Infections can be spread by direct contact with infected animals, indirect contact through objects and surfaces that have come in contact with infected animals, contaminated food or water, and lastly vector borne (CDC 2023).This last method of transmission is typically facilitated by hematophagous ectoparasites feeding first on an infected animal, picking up the pathogen, and passing it to another host through their next feeding. Some examples of these pathogens and their vectors would be plasmodium which causes malaria and is transmitted by mosquitoes, African swine fever through ticks, *Rickettsia prowazekii*, which causes typhus and is spread by fleas, and filarial nematodes which can be transmitted by horn flies (Hibler 1966). To elaborate on the dangers and damages of vector borne diseases, African swine fever was first identified in the early 1900's and believed to have originated in warthogs (Sánchez-Cordón et al. 2018). This disease is a fatal virus transmitted to domestic pigs through soft bodied ticks (Sánchez-Cordón et al. 2018) and in 2021 a loss of 43.46 million domestic pigs was estimated due to disease or culling (You et al. 2021). Pathogens can also be transmitted mechanically by

direct contact. Insects disperse said pathogens by depositing it in food, water, or orifices of their victims. An example of this would be foodborne *E.coli* by cockroaches (Donkor 2020).

An important distinction to note is that not all vectors borne diseases are zoonotic. An example of a deadly vector borne disease within humans would be Malaria. According to the World Health Organization in 2023, the estimated number of deaths caused by Malaria was 619,000 and nearly half of the world's population are at risk of this disease (WHO 2023). However, *Plasmodium falciparum*, the deadliest malaria parasite, can only be found in humans and mosquitoes (WHO 2023). Whereas a vector borne zoonotic disease such as Eastern Equine Encephalitis can affect birds, humans, and horses.

Vector borne zoonotic diseases require a susceptible host and vector. The virus responsible for this ailment is transmitted by different species of mosquitoes and is picked up from its natural reservoir, songbirds, and waterfowl. Reservoirs are long-term hosts in which the virus can be maintained. Reservoirs may show signs of disease; however, it is usually not fatal. On the other hand, in susceptible or dead-end hosts like humans and horses, Eastern Equine Encephalitis is a fatal arboviral disease that can pose life threatening symptoms and permanent neurological damage (Banda and Samanta 2023).

1.1.3 Introduction to Agricultural Ectoparasites

Ectoparasites are organisms that require a host to survive. They are detrimental pests that live on the surface of their hosts, usually only infecting the superficial layers of skin and obtain their sustenance by feeding on the blood, flesh, or hair from their hosts (Muhammad et al. 2021). A host is the organism the ectoparasite feeds on and can range from invertebrates to vertebrates, however this thesis will only focus on humans and cattle as hosts. Some examples of

ectoparasites include fleas, ticks, mites, lice, and even mosquitoes in a broader scope. In agriculture, these pests can have a significant impact on livestock production and their quality of life, leading to decreased productivity, weight loss, depression, and in some cases even death (Nicoletti 2020). The presence of ectoparasites can have serious economic consequences for farmers as well, particularly those raising livestock for meat, dairy, or wool. These pests can severely impact and decrease the quality of life for animals causing tissue damage, irritation, inflammation, loss of weight gain, lameness, anemia, fetal abortions and disease, thereby lowering the quality and quantity of production (Lehmann 1993). If the infestation is significant, damage to the skin and hair of the animal can also decrease the value of hide in the market. In 1992 ectoparasites were estimated to cause over 2.6 billion in damages (Byford et al. 1992). This report was taken over 30 years ago and according to the SmartAsset inflation calculator, since 1992 there has been a 107.89% increase in cumulative inflation. When adjusted for inflation in 2023, the damages are estimated to be over 5.4 billion. These expenses also did not include the costs of insecticides used to treat animals against these pests.

1.1.4 The Importance of Climate Change on Geographical Range of Insects

Climate change is another important factor to take into account when considering ectoparasites. This is because insects are highly adaptable organisms and populations are attuned to environmental temperatures (Skendžić et al. 2021). As temperatures continue to rise, it can be expected that the geographic range of insects will expand. Warmer temperatures can also impact overwintering survival and increase the number of generations within a year. This can lead to higher ectoparasite populations with an expanded range for longer periods of time, resulting in higher instances of disease spread (Colón-González et al. 2021) and larger amounts of stress in people and livestock.

To further gauge the importance of ectoparasites in agriculture in Alabama, one must consider the major problems caused by these pests. This thesis will focus particularly on two different insects within the order Diptera. One is *Culex erraticus,* a mosquito known to transmit zoonotic flaviviruses frequently found in the southeast. The other is *Haematobia irritans irritans,* a cattle parasite. Both of which are hematophagous ectoparasites of medical and veterinary importance and contribute to the spread of disease, economic loss, insecticide resistance, and ultimately can have a negative impact on overall human health.

1.2.1 The Importance of *Culex erraticus*

As mentioned prior, mosquitoes are considered the most dangerous animal to man due to its ability to transmit pathogens. However, as a society our knowledge is limited to those that cause symptomatic diseases. New technology such as Next Generation Sequencing (NGS), has paved a way to exposing and expanding our knowledge of the diverse viruses and pathogens carried by insects (Brinkmann et al. 2016). Previously, these pathogens went "under the radar" if they did not cause significant disease symptoms to warrant intensive investigation. Our ability to identify these underlying pathogens presents new opportunities to examine their interactions with ectoparasites and their hosts.

This thesis will focus mainly on *Culex erraticus,* a small dark mosquito within the *Melanoconion* subgenus. This species is one of the most common of mosquitoes within the southeast US and often found at many enzootic sites (Bingham et al. 2016). They are generalist with a pattern of feeding on avians such as waterfowl and passerines earlier in the season before transitioning to feeding on mammals later on in the season (Oliveira et al. 2011). Avians, especially waterfowl, are often reservoirs for deadly vector borne diseases such as West Nile

Virus and Eastern Equine Encephalitis that can be picked up by *Culex erraticus* (Bingham et al. 2016; Cupp et al. 2004). Importantly, many of these waterfowl are attracted to and gather at fish farms for roosting and foraging opportunities (Callier et al. 2018). Both viruses can also be transmitted to humans and other animals of agricultural importance such as chickens, causing disease and potentially death (Guy et al. 1994). As the geographical range of *Culex erraticus* and human society continue to overlap causing an increase of interactions between vectors and hosts, the potential for major health and food security concerns caused by novel or re-emerging disease outbreaks to occur grows with it.

Fortunately, with the use of NGS, scientists may be able to discover and study pathogens before they make the jump to humans and animals of importance. By making these discoveries before pathogens reach global importance, societies will have more time to create methods and countermeasures to protect against and lessen the effects of future outbreaks.

1.2.2 Distribution & Seasonality of *Culex erraticus*

Mosquitoes within the subgenus *Melanoconion* are generally found in North and South America with most species residing in tropical and subtropical spaces (Subgenus Melanoconion Theobald, 1903). Unlike other species within this subgenus, *Culex erraticus* is more widely distributed yet still considered a rarity in the northeast (Nordgulen et al. 2022). However in recent years, established populations can be found as far north as Ontario, Canada (Hunter et al. 2015) and due to temperature rises, the geographical range of *Culex erraticus* is expected to expand (Hunter et al. 2015). These mosquitoes are also considered the most abundant mosquito species in the southeast US (Bingham et al. 2016).

Culex erraticus is frequently found in a variety of different habitats, but most commonly in freshwater hardwood swamps and marshes (Burkett-Cadena 2013). In states like Alabama, populations fluctuate depending on temperature with peaks in population most likely to occur during the summer and fall months in the southeastern US (Nordgulen et al. 2022). During the colder months, adult females are able to overwinter in caves and hollow trees only to emerge Mid-March once air temperature reaches between 18 to 22 degrees Celsius (Burkett-Cadena et al. 2011). In warmer areas such as southern Florida, *Culex erraticus* do not need to overwinter and have active year-round populations (Bingham et al. 2014).

1.2.3 Characteristics of *Culex erraticus*

Culex mosquitoes can be easily identified by their short palps and rounded abdomen tip. However, it can be rather difficult to distinguish *Culex* mosquitoes down to species. *Culex erraticus* are small and dark in color with broad pale scales lining the edge of their compound eyes (Nordgulen et al. 2022). Mosquitoes have piercing and sucking mouthparts that extend forward (Zahran et al. 2022). These proboscises are utilized by adult females to discreetly take blood meals to provide nutrients for egg development (Nordgulen et al. 2022). Similar to all other mosquitoes, *Culex erraticus* are holometabolous with immature aquatic life stages (Nordgulen et al. 2022). According to the CDC, mosquito eggs can hatch within 48 hours with larvae developing into pupae in as little as 5 days. Adults emerge from pupae in about 2 to 3 days, concluding the life cycle from egg to adult to be a total of 7 to 10 days total depending on temperature(CDC 2022).

1.2.4 The Importance of *Culex erraticus* found in Auburn Fisheries

Female *Culex erraticus* are aggressive biters. As mentioned before, they often feed on avians early in the season before transitioning to feeding on mammals. This allows for a higher chance for these mosquitoes to pick up zoonotic pathogens and pass them to humans. Another concern for *Culex erraticus* is that they are generalist feeders with the ability to take blood meals from avians, reptiles, amphibians, and mammals (Robertson et al. 1993). This is a concern because there is potential for viruses to come from different species that are not yet known.

The Auburn fisheries is a point of interest because of its attraction to waterfowl and proximity to a natural preserve and urban communities. This positioning allows for various species of uncommon wildlife to encounter *Culex erraticus* and a potential for pathogens to be picked up and transmitted to people and or farmed fish. Wherever animals intersect, the possibility of disease transmission is present.

1.3.1 The Importance of *Haematobia irritans irritans*

The horn fly is a hematophagous obligate ectoparasite and is one of, if not, the most important cattle pest in the world. Both male and female horn flies have piercing/ sucking mouthparts that help slash through the thick hide of cattle to feed on their blood. They can blood feed up to 24 to 38 times a day (Harris et al. 1974) with each feedings lasting about 10 minutes (Controlling Horn Flies on Pastured Ca...). Each fly requires an average of 10 μL of blood every day (Russell et al. 2013). When cattle are not available, they will also feed on dogs, goats, and horses. Horn flies often occur in large numbers with intense infestations reaching up to 4,000 flies per cow (Showler et al. 2014). This intimate relationship between cattle and fly, especially when fly populations are high, causes a tremendous amount of stress and annoyance in cattle

resulting in production reduction and major financial loss to producers, increase in disease transmission, and has led to higher rates of insecticide resistance due to insecticide use. These are all alarming issues that have direct and indirect impacts on food production and must be addressed when dealing with feeding the growing human population.

1.3.2 Distribution & Seasonality of *Haematobia irritans irritans*

Haematobia irritans irritans were first introduced in the United States from southern France before 1886. Since their introduction, they have rapidly spread throughout the continental US and Canada. A major contribution to this is due to their ability to travel distances of over 6 kilometers to reach a herd of cattle (Kunz et al. 1983). These pests can now be found globally throughout the Americas, Europe, Asia, northern Africa, and even the Hawaiian islands (Lancaster and Meisch 1986).

Horn fly population numbers/densities rise and fall throughout the year and vary broadly depending on the climate. In cooler areas, there can be anywhere from 6 to 9 generations whereas in warmer more tropical regions one can expect 12 to 14 generations per year (Showler et al. 2014). In semiarid regions of northeastern Brazil, up to 30 generations were recorded indicating that in warmer regions horn flies are a constant obstacle that farmers and cattle face throughout the year (Melo et al. 2020).

In relatively cold regions, horn fly populations substantially die off during the first frost allowing cattle and farmers a period of respite. These populations are renewed the following spring when pupae emerge from overwintering and adults come out of diapause. This is not always the case in warmer temperate and subtropical regions such as Alabama and Florida. In areas that have relatively warm winters, adult horn flies have persisted on cattle, albeit in lower

populations than in the peak times during spring to early fall (Showler et al. 2014). In the southern United States, neighboring states to Alabama such as Florida, and Missouri, have indicated that even during periods of low temperatures adult populations of horn flies have been reported (Koehler et al. n.d.) (Thomas and Morgan 1972). This is a costly problem for farmers in hotter regions such as Alabama, due to the persisting population of horn flies that continue to stress and annoy cattle during warmer winters.

1.3.3 Characteristics of *Haematobia irritans irritans*

Both adult male and female horn flies range from 3 to 5 mm in length, which is about half the size of a common housefly, *Musca domestica* (Mullen and Durden 2009). They are brownish- gray or black in color and have shiny slightly overlapping wings that hold out in an upside down "v" shape atop of their abdomen. There are no major patterns that stand out on the horn fly.

Like other higher classifications of flies, horn flies can be sexed by the distance between their compound eyes. Males have eyes that are closely set together and nearly meet at the apex of their heads. They are thinly set apart by a single row of setae on either side of the midline between each eye, while females have compound eyes that are distinctly set apart (Brewer et al. 2021).

1.3.4 Life cycle of *Haematobia irritans irritans*

Horn flies rarely leave their hosts. Even when disturbed, these flies will simply move to another location on the bovid to continue feeding. Horn flies mate on their hosts for several days after they have emerged as adults from their pupal stage. Females typically only leave their host to oviposit in fresh cow patties (Lancaster and Meisch 1986). Oviposition can occur as early as 3

days after emergence with some 5-day old females ovipositing daily (Krafsur et al. 1992). Horn flies are persistent egg layers and can oviposit at any point in the day and night with the highest number of horn flies laying eggs within the first 2 minutes after a fresh pat of manure is deposited (Sanders and Dobson 1969).

Horn flies are holometabolous which means they undergo metamorphosis. They hatch from eggs as larvae and enter an inactive pupal phase before emerging as adults. Eggs are around 1mm in length and are deposited on the edges and under freshly laid cattle dung. Approximately 14 to 20 eggs are deposited in each batch with older females laying multiple times a day (Lancaster and Meisch 1986; Brewer et al. 2021). A single female horn fly can have approximately 15 batches of eggs totaling up to 360 to 400 eggs within her lifetime (Lancaster and Meisch 1986), this allows for the population to grow rapidly especially in warmer regions. Temperature and humidity are environmental factors that horn flies depend heavily on. During warm seasons, eggs can hatch in as little as 11 hours (McLintock and Depner 1957) and larvae can reach adulthood in as little as 7 to 11 days at optimal temperatures (Melo et al. 2020) allowing for more generations in a year and higher populations.

1.3.5 Economic injury on Agriculture Caused by *Haematobia irritans irritans*

In an economic impact study of cattle parasites in Brazil of 2014, scientists estimated a combined $13.96 billion loss. Of that grand total, $3.24 billion was caused by horn flies alone (Grisi et al. 2014). In the United States, the loss from horn flies was estimated to be more than $730 million a year, however these estimates were taken from a study in 1991 and are presumed to have increased over the last decades due to inflation and climate change.

Horn flies can be found year round in certain areas of the world; in some cases when cattle are not a viable host, populations of these pests can be found feeding on other livestock

and domestic animals such as goats, horses, and dogs (Brewer et al. 2021). These pests can infest every part of the cattle's body. They are typically found clustered along the backs, sides, and withers of cattle. As the days progressively get hotter, horn flies will migrate to the belly region of the host to continue feeding (McLintock and Depner 1957). It has been widely accepted that the economic injury and treatment level per cow is 200 flies per head of cattle (Gordon et al. 2008). This means that the lowest population density of horn flies that will cause economic damage or justify the cost of control is 200 flies per cattle (Stern et al., 1959). However, there have been reports of heavy infestations resulting in over 4,000 flies per cattle (Showler et al. 2014).

The direct economic impacts of horn fly feeding on cattle are seen through yield loss. Some examples of this include decreased weight gain, weaning weight, milk production, and feed efficiency (Brewer et al. 2021). These losses are attributed to blood loss, stress, and annoyance due to irritation. Cattle can often be seen flipping their heads, stomping, and flicking their tails to ward off flies. These actions cost energy and take away time from feeding leading to lower weight gain. In a study on cattle growth treated with insecticide against horn flies, those who received treatment showed an increase in weight gain when compared to cattle that did not receive insecticide treatment (Haufe 1982). Ectoparasites can also indirectly affect financial gain by increasing producer costs. These impacts are manifested through disease and insecticide use.

Aside from stress and direct injury, horn flies are speculated to be vectors for pathogens such as bovine leukemia virus, fungus, and other harmful bacteria (Panei et al. 2019). Horn flies can introduce unwanted bacteria to sensitive parts of cattle such as the mammary glands causing infection and inflammation known as mastitis. This disease is one of the main causes for reduced

milk yield and quality leading to over $200 loss per cow annually in the dairy industry (Jeffrey F Keown 2007).

Insecticides and labor associated with application on cattle against horn flies are another indirect cost. To assess their expenses on insecticide use in 2016, cow-calf producers in Tennessee and Texas were surveyed and an average of $9.50/ head and $12.40/head was spent respectively (Smith et al. 2022). These prices can easily add up and be a financial burden for farmers. To offset these costs, producers are willing to pay premium prices for bulls resistant to horn flies (Mckay et al. 2003).

1.3.6 Management Methods of *Haematobia irritans irritans*

Numerous amounts of products, traps, and strategies have been developed over the years to attempt to control horn fly populations. Producers have utilized management methods including chemicals and non-chemical strategies consisting of physical traps, biological control agents, and pasture management (Brewer et al. 2021). All of which have their own pros and cons.

One of the most common methods to control horn flies is utilizing a variety of insecticides with different modes of application. Some methods require daily application which can be fulfilled with self-treating methods such as dust bags and back rubbers. These methods are most successful when animals are forced to pass through these devices to access water or other essentials, however they are more practical for smaller herds (Williams et al. 1985). On the other hand, whole animal sprays are applied every 2 to 4 weeks. Application is not as frequent; however, this method is very labor intensive since the animals need to be gathered and moved (Williams et al. 1985). Large herds of cattle and herds that graze on expansive rangelands may

not be well adapted to human care (Creamer and Horback 2021), therefore, spraying individual cattle in large herds may also be difficult. To counter these challenges, ear tags impregnated with insecticides were developed so that cattle could self-apply on the pasture. The downside is the continued use of these artificial chemicals, causing new obstacles to arise regarding insecticide resistance.

1.3.7 Insecticide resistance

Insecticide resistance is the reduction of susceptibility in a pest population when exposed to insecticides that have previously been more effective at controlling said population. It is also expected to develop rapidly for a number of reasons including intensity at which insecticides are used, intrinsic factors of pest populations such as genetic traits, and extrinsic ones due to incorrect use of insecticides by management (Denholm and Rowland 1992; Byford et al. 1999).

There are a variety of ways in which resistance can develop. The most common methods include behavioral and genetic evolution (Oyarzún et al. 2008). Genetic evolution can be sped up by quick generation times. The more generations there are, the more likely insecticide resistance genes will emerge in a population (Insecticide Resistance Action Committee n.d.). Horn flies have demonstrated resistance to various insecticides including organophosphates, pyrethroids, and diflubenzuron, which may be attributed to their many generations in warmer regions (Oyarzún et al. 2008).

1.3.8 Introduction to Saliva Vaccine

Due to high management costs and the growing rate of insecticide resistance, scientists have pioneered new approaches to combat growing horn fly populations. One of these novel methods is the use of vaccines. Advantages of vaccine use is due to the long periods of protection and high species specificity they provide. These advantages aid in slowing the

development of resistance (Pruett 1999), while also theoretically combatting horn fly populations.

One promising vaccine was developed at Auburn University by Dr. Mary Cupp. This vaccine targets thrombostasin (TS), a salivary protein that inhibits thrombin in cattle (Cupp et al. 1998; Zhang et al. 2002; Cupp et al. 2004; Cupp et al. 2000; Cupp et al. 2010). Thrombin is an enzyme found in blood plasma that is essential for hemostasis, otherwise known as the stopping of the flow of blood (Narayanan 1999). This protein turns fibrinogen into insoluble fibrin clots, which is vital for coagulation (Al-Amer 2022). By developing and utilizing thrombostasins, horn flies are able to blood feed unabated on their hosts.

Unlike most other hematophagous arthropods, horn flies have lower diversity in anti-hemostatic compounds (Cupp et al. 1998). In fact, thrombostasin seems to be the sole salivary protein responsible for anti-clotting in horn flies (Zhang et al. 2002). Due to this unique circumstance, the creation of a vaccine targeting TS, was considered a great opportunity to interrupt the feeding ability of horn flies without any off-target effects (Oyarzún et al. 2008). This is because thrombostasins is a species-specific protein and the sole method of anti-clotting horn flies use. By injecting cattle with the recombinant TS protein, cattle can develop antibodies against TS and inhibit the anti-clotting properties of the horn fly saliva preventing the flies from feeding as efficiently. By doing so, this would alter the feeding behavior of the horn fly and interrupt their life cycle by impairing reproductive fecundity.

Dr. Mary Cupp first discovered thrombostasins in horn fly salivary glands in 1998 (Cupp et al. 1998) and purified through high performance liquid chromatography (Zhang et al. 2002). Since then, a viable recombinant protein vaccine has been developed, tested, and improved. This was done by expressing the thrombostasin isoforms in E. coli using the pET30a expression

plasmid within cloning site (NdeI-HIndIII). The proteins were then purified via affinity columns using the C-His6 tag. Initial experiments consisted of immunizing both cattle and rabbits with a recombinant thrombostasin vaccine and exposing them to lab reared horn flies. Vaccinated animals were tested for IgG titers to prove the development of antibodies against thrombostasin. Later, exposing the vaccinated animals to horn flies provided an idea that feeding behavior of the horn flies was impaired due to antibody response within the cattle. These tests were met with positive results and demonstrated that horn flies feeding on immunized hosts received smaller blood meals (Cupp et al. 2004) than those that fed on non-immunized hosts. It was also noted that a reduction in blood meals caused a delay in egg development, which could affect fecundity and ultimately impact horn fly populations (Cupp et al. 2004). These experiments proved promising for a novel method to combat horn fly populations.

It was later discovered that horn flies carry multiple isoforms of thrombostasin within their population (Cupp et al. 2010). This meant horn flies carrying different isoforms of thrombostasin would not be affected by the vaccine. To address this, the vaccine became a cocktail of recombinant thrombostasin isoforms and requires further testing before it can be authorized for commercial use.

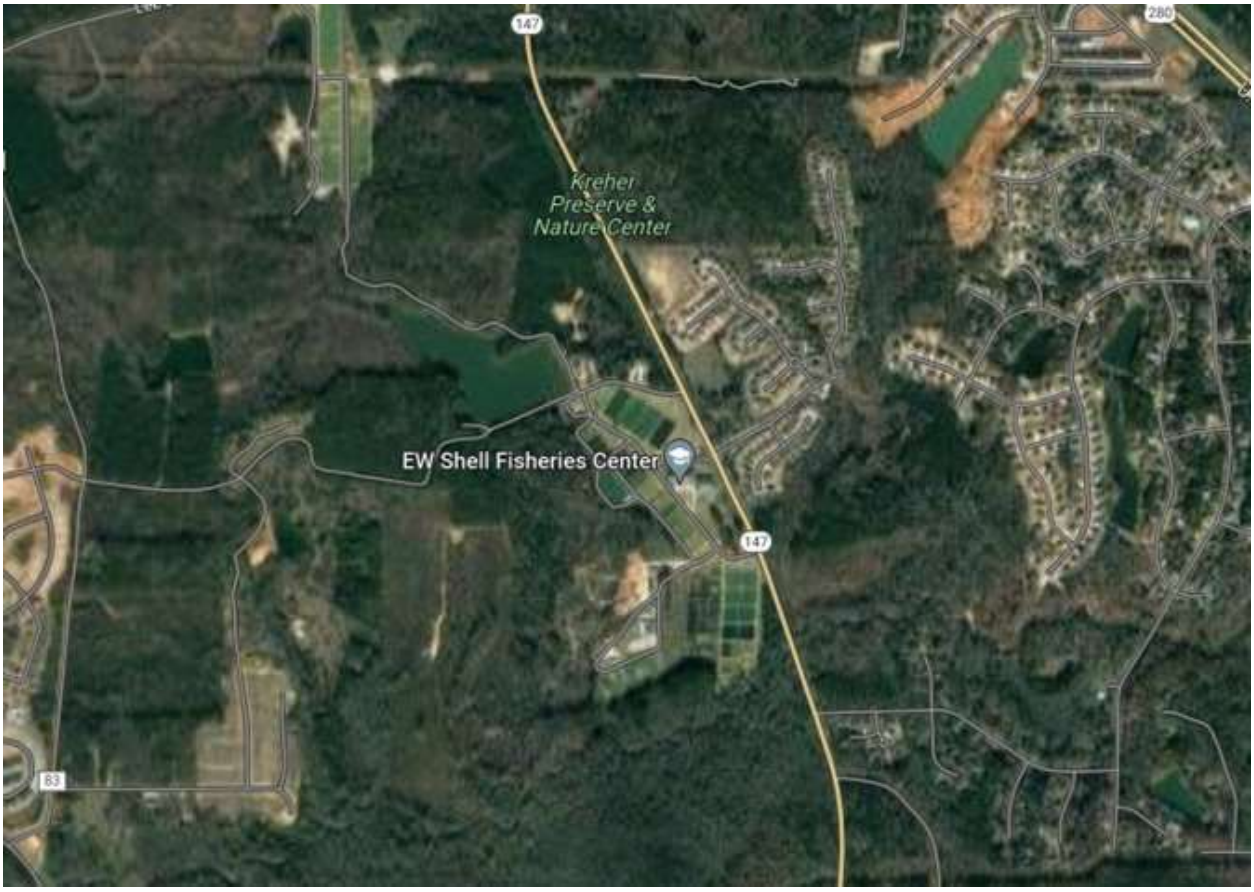

Figure 1.1 Map Depicting Location of Auburn Fisheries. The Auburn fisheries is located near a wildlife preserve.

Figure 2.1 Horn Flies on Auburn University cattle. This figure shows the high number of horn flies that can infest smaller and better managed cattle herds.

References

"8 Zoonotic Diseases Shared Between Animals and People of Most Concern in the U.S." 2019. CDC. September 9, 2019. https://www.cdc.gov/media/releases/2019/s0506-zoonotic-diseases-shared.html.

Al-Amer, Osama M. 2022. "The Role of Thrombin in Haemostasis." Blood Coagulation & Fibrinolysis: An International Journal in Haemostasis and Thrombosis 33 (3): 145–48.

Al Hajjar, Sami, and Kenneth McIntosh. 2010. "The First Influenza Pandemic of the 21st Century." Annals of Saudi Medicine 30 (1): 1–10.

Banda, Cecilia, and Debopam Samanta. 2023. Eastern Equine Encephalitis. StatPearls Publishing.

Barbier, Edward B. 2021. "Habitat Loss and the Risk of Disease Outbreak." Journal of Environmental Economics and Management 108 (July): 102451.

Baquedano, Felix G., Yacob Abrehe Zereyesus, Constanza Valdes, and Kayode Ajewole. 2021. "Global Food Insecurity Grows in 2022 Amid Backdrop of Higher Prices, Black Sea Conflict." *Amber Waves*, September 7, 2021. https://www.ers.usda.gov/amber-waves/2021/september/food-insecurity-to-increase-in-2021-at-a-higher-rate-in-lower-income-countries/.

Bingham, Andrea M., Nathan D. Burkett-Cadena, Hassan K. Hassan, Christopher J. W. McClure, and Thomas R. Unnasch. 2014. "Field Investigations of Winter Transmission

of Eastern Equine Encephalitis Virus in Florida." The American Journal of Tropical Medicine and Hygiene 91 (4): 685–93.

Bingham, Andrea M., Nathan D. Burkett-Cadena, Hassan K. Hassan, and Thomas R. Unnasch. 2016. "Vector Competence and Capacity of Culex Erraticus (Diptera: Culicidae) for Eastern Equine Encephalitis Virus in the Southeastern United States." Journal of Medical Entomology 53 (2): 473–76.

Brewer, Gary J., Dave J. Boxler, Luísa N. Domingues, Rebecca T. Trout Fryxell, Chris Holderman, Kelly M. Loftin, Erika Machtinger, Brandon Smythe, Justin L. Talley, and Wes Watson. 2021. "Horn Fly (Diptera: Muscidae)—Biology, Management, and Future Research Directions." Journal of Integrated Pest Management 12 (1). https://doi.org/10.1093/jipm/pmab019.

Brinkmann, Annika, Andreas Nitsche, and Claudia Kohl. 2016. "Viral Metagenomics on Blood-Feeding Arthropods as a Tool for Human Disease Surveillance." International Journal of Molecular Sciences 17 (10). https://doi.org/10.3390/ijms17101743.

Burkett-Cadena, Nathan D. 2013. Mosquitoes of the Southeastern United States. University of Alabama Press.

Burkett-Cadena, Nathan D., Gregory S. White, Micky D. Eubanks, and Thomas R. Unnasch. 2011. "Winter Biology of Wetland Mosquitoes at a Focus of Eastern Equine Encephalomyelitis Virus Transmission in Alabama, USA." Journal of Medical Entomology 48 (5): 967–73.

Byford, R. L., M. E. Craig, and B. L. Crosby. 1992. “A Review of Ectoparasites and Their Effect on Cattle Production.” Journal of Animal Science 70 (2): 597–602.

Byford, R. L., M. E. Craig, S. M. DeRouen, M. D. Kimball, D. G. Morrison, W. E. Wyatt, and L. D. Foil. 1999. “Influence of Permethrin, Diazinon and Ivermectin Treatments on Insecticide Resistance in the Horn Fly (Diptera: Muscidae).” International Journal for Parasitology 29 (1): 125–35; discussion 137–38.

Callier, Myriam D., Carrie J. Byron, David A. Bengtson, Peter J. Cranford, Stephen F. Cross, Ulfert Focken, Henrice M. Jansen, et al. 2018. “Attraction and Repulsion of Mobile Wild Organisms to Finfish and Shellfish Aquaculture: A Review.” *Reviews in Aquaculture* 10 (4): 924–49.

CDC. 2022. “Life Cycle of Culex Species Mosquitoes.” Centers for Disease Control and Prevention. July 12, 2022. https://www.cdc.gov/mosquitoes/about/life-cycles/culex.html.

Colón-González, Felipe J., Maquins Odhiambo Sewe, Adrian M. Tompkins, Henrik Sjödin, Alejandro Casallas, Joacim Rocklöv, Cyril Caminade, and Rachel Lowe. 2021. “Projecting the Risk of Mosquito-Borne Diseases in a Warmer and More Populated World: A Multi-Model, Multi-Scenario Intercomparison Modelling Study.” The Lancet. Planetary Health 5 (7): e404–14.

Creamer, Maggie, and Kristina Horback. 2021. “Researching Human-Cattle Interaction on Rangelands: Challenges and Potential Solutions.” *Animals : An Open Access Journal from MDPI* 11 (3). https://doi.org/10.3390/ani11030725.

"Controlling Horn Flies on Pastured Cattle in Nebraska." n.d. UNL Beef. Accessed June 3, 2023. https://beef.unl.edu/beefwatch/controlling-horn-flies-pastured-cattle-nebraska.

Cupp, E. W., M. S. Cupp, J. M. Ribeiro, and S. E. Kunz. 1998. "Blood-Feeding Strategy of Haematobia Irritans (Diptera: Muscidae)." Journal of Medical Entomology 35 (4): 591–95.

Cupp, E. W., K. J. Tennessen, W. K. Oldland, H. K. Hassan, G. E. Hill, C. R. Katholi, and T. R. Unnasch. 2004. "Mosquito and Arbovirus Activity During 1997-2002 in a Wetland in Northeastern Mississippi." Journal of Medical Entomology 41 (3): 495–501.

Cupp, Mary S., Eddie W. Cupp, Christine Navarre, Dunhua Zhang, Xin Yue, Latora Todd, and Victor Panangala. 2010. "Salivary Gland Thrombostasin Isoforms Differentially Regulate Blood Uptake of Horn Flies Fed on Control- and Thrombostasin-Vaccinated Cattle." Journal of Medical Entomology 47 (4): 610–17.

Cupp, Mary S., E. W. Cupp, Christine Navarre, Nancy Wisnewski, Kevin S. Brandt, Gary M. Silver, Dunhua Zhang, and Victor Panangala. 2004. "Evaluation of a Recombinant Salivary Gland Protein (thrombostasin) as a Vaccine Candidate to Disrupt Blood-Feeding by Horn Flies." Vaccine 22 (17-18): 2285–97.

Denholm, I., and M. W. Rowland. 1992. "Tactics for Managing Pesticide Resistance in Arthropods: Theory and Practice." Annual Review of Entomology 37: 91–112.

Donkor, Eric S. 2020. "Cockroaches and Food-Borne Pathogens." Environmental Health Insights 14 (April): 1178630220913365.

Dotis, J., and E. Roilides. 2009. “H1N1 Influenza A Infection.” Hippokratia 13 (3): 135–38.

Ericksen, Polly J., John S. I. Ingram, and Diana M. Liverman. 2009. “Food Security and Global Environmental Change: Emerging Challenges.” *Environmental Science & Policy* 12 (4): 373–77.

Food and Agriculture Organization of the United Nations, International Fund for Agricultural Development, United Nations Children’s Fund, United Nations World Food Programme, and World Health Organization. 2023. “The State of Food Security and Nutrition in the World 2023.” *The State of Food Security and Nutrition in the World*. https://doi.org/10.4060/cc3017en.

Gordon, D. V., W. D. Haufe, and K. K. Klein. 2008. “Determination of Economic Thresholds for Horn Fly Control in Western Canada: A Farm Level Simulation Approach.” Canadian Journal of Agricultural Economics 32 (2): 399–421.

Grisi, Laerte, Romário Cerqueira Leite, João Ricardo de Souza Martins, Antonio Thadeu Medeiros de Barros, Renato Andreotti, Paulo Henrique Duarte Cançado, Adalberto Angel Pérez de León, Jairo Barros Pereira, and Humberto Silva Villela. 2014. “Reassessment of the Potential Economic Impact of Cattle Parasites in Brazil.” Revista Brasileira de Parasitologia Veterinaria = Brazilian Journal of Veterinary Parasitology: Orgao Oficial Do Colegio Brasileiro de Parasitologia Veterinaria 23 (2): 150–56.

Guy, J. S., H. J. Barnes, and L. G. Smith. 1994. “Experimental Infection of Young Broiler Chickens with Eastern Equine Encephalitis Virus and Highlands J Virus.” Avian Diseases 38 (3): 572–82.

Haider, Najmul, Peregrine Rothman-Ostrow, Abdinasir Yusuf Osman, Liã Bárbara Arruda, Laura Macfarlane-Berry, Linzy Elton, Margaret J. Thomason, et al. 2020. “COVID-19-Zoonosis or Emerging Infectious Disease?” Frontiers in Public Health 8 (November): 596944.

Harris, R. L., J. A. Miller, and E. D. Frazar. 1974. “Horn Flies and Stable Flies: Feeding Activity.” Annals of the Entomological Society of America 67 (6): 891–94.

Haufe, W. O. 1982. “GROWTH OF RANGE CATTLE PROTECTED FROM HORN FLIES (Haematobia Irritans) BY EAR TAGS IMPREGNATED WITH FENVALERATE.” Canadian Journal of Animal Science 62 (2): 567–73.

Hibler, C. P. 1966. “Development of Stephanofilaria Stilesi in the Horn Fly.” *The Journal of Parasitology* 52 (5): 890–98.

“Horn Fly - Haematobia Irritans Irritans (Linnaeus).” n.d. Accessed June 5, 2023. https://entnemdept.ufl.edu/creatures/livestock/flies/horn_fly.htm.

Hunter, F. F., J. Causarano, A. Gasparotto, and B. V. Giordano. 2015. “Establishment of Culex (Melanoconion) Erraticus (Diptera: Culicidae) in Southern Ontario, Canada.” Journal of Medical Entomology 52 (3): 509–12.

Insecticide Resistance Action Committee. n.d. “Insecticide Resistance: Causes and Action.” USDA.gov. Accessed June 18, 2023. https://www.nifa.usda.gov/sites/default/files/resources/Insecticide%20resistance.pdf.

Keown, J.f. and Kononoff, P. J. 2007. “How to Interpret the DHIA-520 Somatic Cell Count Profile.” NEBGuide. https://extensionpublications.unl.edu/assets/pdf/g1656.pdf.

Koehler, P. G., J. F. Butler, and P. E. Kaufman. n.d. “Horn Flies1.” http://www.floridacattleranch.org/ifas_horn_flies.pdf.

Krafsur, E. S., A. L. Rosales, J. F. Robison-Cox, and J. P. Turner. 1992. “Age Structure of Horn Fly (Diptera: Muscidae) Populations Estimated by Pterin Concentrations.” *Journal of Medical Entomology* 29 (4): 678–86.

Kunz, S. E., H. G. Kinzer, and J. A. Miller. 1983. “Areawide Cattle Treatments on Populations of Horn Flies (Diptera: Muscidae).” Journal of Economic Entomology 76 (3): 525–28.

Lancaster, J. L., Jr, and M. V. Meisch. 1986. “Arthropods in Livestock and Poultry Production.” In Arthropods in Livestock and Poultry Production. Ellis Horwood.

Lehmann, T. 1993. “Ectoparasites: Direct Impact on Host Fitness.” Parasitology Today 9 (1). https://doi.org/10.1016/0169-4758(93)90153-7.

“Malaria.” 2023. Accessed June 5, 2023. https://www.who.int/news-room/fact-sheets/detail/malaria.

Mckay, Lettie, Karen L. Delong, David B. Taylor, and Research Entomologist. 2003. “Cow-Calf Producers’ Willingness to Pay for Bulls Resistant to Horn Flies, Haematobia Irritans (L.) (Diptera: Muscidae).” 2003. https://extension.tennessee.edu/publications/Documents/W821.pdf.

McLintock, J., and K. R. Depner. 1957. “Preliminary Attempts to Establish a Laboratory Colony of the Horn Fly, Siphona Irritans (L.) (Diptera: Muscidae).” The Journal of Parasitology 43 (2): 209–12.

Melo, Lídio Ricardo Bezerra, Márcia Alves Medeiros, Lucas Alencar Fernandes Beserra, Antônio Thadeu Medeiros Barros, Franklin Riet-Correa, Sérgio Santos Azevedo, and Vinícius Longo Ribeiro Vilela. 2020. “Development and Number of Generations of Haematobia Irritans (Diptera: Muscidae) in Bovine Fecal Masses in the Semiarid Region of Brazil.” Veterinary Parasitology, Regional Studies and Reports 20 (April): 100411.

Mena, Ignacio, Martha I. Nelson, Francisco Quezada-Monroy, Jayeeta Dutta, Refugio Cortes-Fernández, J. Horacio Lara-Puente, Felipa Castro-Peralta, et al. 2016. “Origins of the 2009 H1N1 Influenza Pandemic in Swine in Mexico.” eLife 5 (June). https://doi.org/10.7554/eLife.16777.

Muhammad, Ali, Rida Bashir, Majid Mahmood, Muhammad Sohail Afzal, Sami Simsek, Usman Ayub Awan, Mobushir Riaz Khan, Haroon Ahmed, and Jianping Cao. 2021. “Epidemiology of Ectoparasites (Ticks, Lice, and Mites) in the Livestock of Pakistan: A Review.” Frontiers in Veterinary Science 8 (December): 780738.

Mullen, Gary R., and Lance A. Durden. 2009. Medical and Veterinary Entomology. Academic Press.

Narayanan, S. 1999. “Multifunctional Roles of Thrombin.” *Annals of Clinical Laboratory Science* 29 (4): 275–80.

Nicoletti, Marcello. 2020. “Chapter Seven - New Solutions Using Natural Products.” In Insect-Borne Diseases in the 21st Century, edited by Marcello Nicoletti, 263–351. Academic Press.

Nordgulen, Mary, Nathan Burkett-Cadena, and Derrick Mathias. 2022. “A Mosquito Culex Erraticus (Dyar and Knab, 1906) (Insecta: Diptera: Culicidae: Culicini)1.” https://doi.org/10.32473/edis-IN1357-2022.

Oldroyd, Harold. 2023. “Dipteran.” In Encyclopedia Britannica. https://www.britannica.com/animal/dipteran.

Oliveira, Ana, Charles R. Katholi, Nathan Burkett-Cadena, Hassan K. Hassan, Sibylle Kristensen, and Thomas R. Unnasch. 2011. “Temporal Analysis of Feeding Patterns of Culex Erraticus in Central Alabama.” Vector Borne and Zoonotic Diseases 11 (4): 413–21.

Oyarzún, M. P., A. Quiroz, and M. A. Birkett. 2008. “Insecticide Resistance in the Horn Fly: Alternative Control Strategies.” Medical and Veterinary Entomology 22 (3): 188–202.

Panei, Carlos Javier, Alejandra Edith Larsen, Nadia Analía Fuentealba, German Ernesto Metz, María Gabriela Echeverría, Cecilia Mónica Galosi, and Alejandro Rafael Valera. 2019. “Study of Horn Flies as Vectors of Bovine Leukemia Virus.” *Open Veterinary Journal* 9 (1): 33–37.

Pruett, J. H. 1999. “Immunological Control of Arthropod Ectoparasites--a Review.” International Journal for Parasitology 29 (1): 25–32.

Robertson, L. C., S. Prior, C. S. Apperson, and W. S. Irby. 1993. “Bionomics of Anopheles Quadrimaculatus and Culex Erraticus (Diptera: Culicidae) in the Falls Lake Basin, North Carolina: Seasonal Changes in Abundance and Gonotrophic Status, and Host-Feeding Patterns.” Journal of Medical Entomology 30 (4): 689–98.

Russell, Richard C., Domenico Otranto, and Richard L. Wall. 2013. The Encyclopedia of Medical and Veterinary Entomology. CABI.

Sánchez-Cordón, P. J., M. Montoya, A. L. Reis, and L. K. Dixon. 2018. “African Swine Fever: A Re-Emerging Viral Disease Threatening the Global Pig Industry.” Veterinary Journal 233 (March): 41–48.

Sanders, D. P., and R. C. Dobson. 1969. “Contributions to the Biology of the Horn Fly12.” Journal of Economic Entomology 62 (6): 1362–66.

Showler, Allan T., Weste L. A. Osbrink, and Kimberly H. Lohmeyer. 2014. “Horn Fly, Haematobia Irritans Irritans (L.), Overwintering.” International Journal of Tropical Insect Science 6 (May). https://doi.org/10.4137/IJIS.S15246.

Simonsen, Lone, and Cecile Viboud. 2021. “A Comprehensive Look at the COVID-19 Pandemic Death Toll.” eLife. https://doi.org/10.7554/eLife.71974.

Skendžić, Sandra, Monika Zovko, Ivana Pajač Živković, Vinko Lešić, and Darija Lemić. 2021. “The Impact of Climate Change on Agricultural Insect Pests.” Insects 12 (5). https://doi.org/10.3390/insects12050440.

Smith, K. V., K. L. DeLong, A. P. Griffith, C. N. Boyer, C. C. Martinez, S. M. Schexnayder, and R. T. Trout Fryxell. 2022. “Costs of Horn Fly (Diptera: Muscidae) Control for Cow-Calf Producers in Tennessee and Texas, 2016.” Journal of Economic Entomology 115 (1): 371–80.

Stern, Vmrf, R. Smith, Robert Van den Bosch, Kenneth Hagen, and Others. 1959. “The Integration of Chemical and Biological Control of the Spotted Alfalfa Aphid: The Integrated Control Concept.” *Hilgardia* 29 (2): 81–101.

“Subgenus Melanoconion Theobald, 1903.” n.d. Accessed June 12, 2023. https://mosquito-taxonomic-inventory.myspecies.info/subgenus-emmelanoconionem-theobald-1903.

Thomas, G. D., and C. E. Morgan. 1972. “Field-Mortality Studies of the Immature Stages of the Horn Fly in Missouri.” Environmental Entomology 1 (4): 453–59.

Williams, Ralph E., Robert D. Hall, Alberto B. Broce, and Philip J. Scholl, eds. 1985. Livestock Entomology. Wiley-interscience.

You, Shibing, Tingyi Liu, Miao Zhang, Xue Zhao, Yizhe Dong, Bi Wu, Yanzhen Wang, Juan Li, Xinjie Wei, and Baofeng Shi. 2021. “African Swine Fever Outbreaks in China Led to Gross Domestic Product and Economic Losses.” Nature Food 2 (10): 802–8.

Zahran, Nagwan, Sameh Sawires, and Ali Hamza. 2022. “Piercing and Sucking Mouth Parts Sensilla of Irradiated Mosquito, Culex Pipiens (Diptera: Culicidae) with Gamma Radiation.” Scientific Reports 12 (1): 17833.

Zhang, D., M. S. Cupp, and E. W. Cupp. 2002. “Thrombostasin: Purification, Molecular Cloning and Expression of a Novel Anti-Thrombin Protein from Horn Fly Saliva.” Insect Biochemistry and Molecular Biology 32 (3): 321–30.

“Zoonotic Diseases.” 2021. May 10, 2023. https://www.cdc.gov/onehealth/basics/zoonotic-diseases.html.

Chapter 2

Horn Fly Feeding is Inhibited by Vaccination Against Thrombostasins in Cattle

2.1 Abstract

Horn flies are hematophagous ectoparasites that decrease milk and meat production in dairy and beef cattle. They are a significant economically detrimental pest within the cattle industry and are a threat to food security as the human population continues to grow. Traditional management methods such as insecticides have increasingly lost their potency due to the development of resistance to these agents in horn fly populations. New tools are needed in order to control these pests. To address this critical need, an experimental vaccine has been developed which targets thrombostasins. To date, thrombostasins are the only known salivary protein family secreted by utilized by horn flies that can block the host clotting cascade. Thus, vaccinating cattle against thrombostasins is a potential strategy to interrupt horn fly feeding behavior and ultimately to impair the horn fly life cycle. To determine the clinical efficacy of this experimental vaccine a cohort of 4 vaccinated and 5 control heifers were created. Acute feeding challenges were conducted to measure the impact of vaccination status on horn fly midgut blood volume as a marker for feeding behavior and chronic feeding challenges were conducted to measure the impact of vaccination status on clinical dermatitis score in skin biopsy samples as a marker for the impact of fly biting on the host and on.fly mortality as an indirect marker of fly energy balance. These tests demonstrated that starved horn flies were unable to feed as efficiently on vaccinated cattle compared to unvaccinated controls. Starved flies that were allowed to feed on vaccinated heifers for 25 minutes displayed a 46% lower blood meal volume in their midguts compared to horn flies feeding on unvaccinated cattle for the same

duration. After a 3-day exposure to vaccinated or unvaccinated cattle, caged horn flies feeding on unvaccinated cattle had a 24% higher survivability than those that fed on vaccinated. This difference in mortality is consistent with decreased feeding in flies exposed to vaccinated hosts indicating vaccination against thrombostasins can negatively impact both horn fly behavior and physiology. These results suggest vaccinating cattle against thrombostasins inhibits horn fly feeding in a way that could alter the fly life cycle. These data provide a rational for commercially licensing this experimental vaccine. Doing so would providebe a new tool for farmers to help manage horn fly populations and increase cattle wellbeing.

2.2 Introduction

Causing losses to the cattle industry totaling billions of dollars annually, *Haematobia irritans irritans,* continues to be one of the most economically detrimental pests to manage across the world. These blood-sucking arthropods decrease milk and meat production by not only reducing weight gain and feed efficiency, but also through devaluation of byproducts, i.e. damaging leather quality (Byford et al. 1999; Guglielmone et al. 1999; Brewer et al. 2021). An economic impact study on cattle parasites conducted in Brazil during the 2014 growing season indicated that $3.24 billion in loss was attributable to horn flies alone (Grisi et al. 2014). Whereas in 1991, the United States recorded a loss of more than $730 million a year caused by horn flies, which translates to $1.65 billion in 2023 values according to the CPI inflation calculator. These are direct costs that do not consider the expenses for insecticides and other management investments utilized to control horn fly populations.

The economic threshold whereby horn fly predation induces negative effects on the host and resulting economic losses is as low as 200 flies per head; however, depending upon the environment, the number of horn flies simultaneously feeding on the bovine host can exceed

4,000 flies per animal illustrating the magnitude of the problem caused by horn fly predation (Gordon et al. 2008; Showler et al. 2014). Unfortunately, horn flies are difficult to manage due to their ability to quickly develop tolerance against most classes of insecticides and apparent resistance to new products (Oyarzún et al. 2008). In warmer climates where temperatures remain high enough to preclude overwintering, horn flies may pose a problem to producers and cattle year-round (Thomas and Morgan 1972).

Both male and female adult horn flies are obligate hematophagous ectoparasites of cattle. In laboratory reared settings, female horn flies feed an average of at least 10 times a day (Harris et al. 1974), whereas in the wild, they have been observed to feed between 20 to 38 times (Lancaster and Meisch 1986). Horn flies rarely leave their host, except when females lay eggs in fresh cow manure. A single female can lay up to 400 eggs within her lifetime and eggs can mature to adults in as little as 7 to 11 days at optimal ambient temperatures (Lancaster and Meisch 1986; Melo et al. 2020).

To aid in their feeding behaviors, horn flies have saw-like proboscises through which they also secrete anticoagulation factors within their saliva that allow flies to feed on cattle unabated. This anticoagulation factor has been isolated and identified as a protein isoform within the thrombostasins family. Thrombostasins inhibit host circulating thrombin from hydrolyzing fibrinogen and thus prevents the clotting cascade (Cupp et al. 2000). After this initial discovery, multiple isoforms of thrombostasins have been identified within horn fly saliva. The most prominent are TB8, TS9, and TS10 (Cupp et al. 2010).

Unlike most other hematophagous arthropods, horn flies are unique in that the possess a limited array of anti-hemostasis factors within their saliva and this may cause them to be susceptible to host hemostasis (Cupp et al. 2004).For this reason, multiple vaccines consisting of

different isoforms of recombinant thrombostasins have been created and tested in an effort to exploit this apparent vulnerability (Cupp et al. 2010). These experimental vaccines elicited an immune response in cattle and have shown varying ability to alter the feeding behavior of horn flies (Cupp et al. 2010). These studies led to the development of the current novel experimental vaccine, which is a cocktail of full length, purified recombinant TS8, TS9, and TS10. The efficacy of the experimental vaccine is not yet known, however a safety test in cattle at New Mexico State University demonstrated an immune response with robust antibody titers against the thrombostasins suggesting that it could be efficacious against horn fly predation (Unpublished data)

Such robust IgG responses signify that the host's immune system is responding to the antigen within the vaccine and producing antibodies against thronbostasins. However, the efficacy of this novel experimental vaccine against horn flies is still unknown. If the vaccine is viable, the antibodies should inhibit thrombostasins secreted within horn fly saliva resulting in altered fly feeding behavior and negative effects on fly physiology. The purpose of this study is to evaluate this vaccine by comparing its effects on horn fly feeding behavior through multiple assays meant to assess the impact of vaccination status on horn fly feeding behavior and physiology and on the clinical effect of horn fly exposure has on the bovine host. To measure the physiological differences between fly feeding on vaccinated or unvaccinated cattle, an assay comparing the blood volume of midguts following 25-minute feeding bouts was conducted along with a mortality test. Additionally, to gauge the clinical impact of horn fly exposure on cattle, a scoring system for dermatitis was developed and utilized to assess fly biting intensity between vaccinated and unvaccinated cattle. We hypothesize that if the vaccine will prevent the horn flies from feeding as efficiently, then the horn flies feeding on vaccinated cattle will have lower blood

midgut volume, higher mortality, and dermatitis of hosts will be different based on vaccination status.

2.3 Materials & Methods

2.3.0 Live animal husbandry

All animal activities were approved by the Auburn University Institutional Animal Care and Use Committee, approval number PRN 2020-3777. During this experiment, 9 weaned prepubescent heifers of similar age and weight were individually housed in 8' x 8' penned enclosures on a concrete shaded paddock topped with cardboard shavings. These animals were fed 5lbs of concentrate once a day, hay twice daily, and had ad libitum access to water. Nutritional management was sufficient to meet the NRC requirements for growing heifers (NRC, 2016). Animals were maintained on pasture when not used for fly challenges. 5 heifers were used as controls and the remaining 4 were vaccinated with the experimental vaccine. Vaccinated animals received a priming dose of the experimental vaccine followed by a booster dose three weeks later via I.M. injection. The doses consist of 3 different full length, purified recombinant thrombostasins isoforms: Ts8, Ts9, Ts10, suspended in saline with ENABL. ENABL is a proprietary cattle vaccine adjuvant that will help stabilize the recombinant proteins as well as irritate the immune system. Control animals received phosphate buffered saline (PBS).

The live animal experiments were conducted in two phases. Phase one was conducted in unvaccinated animals to assess fly cage design and to determine the optimal caged fly exposure that induces dermatitis in cattle. Phase 2 was conducted was conducted using cohorts of control and vaccinated animals to test the efficacy of the vaccine using an acute duration of fly exposure to assess midgut blood meal volumes and a chronic caged fly exposure to determine the impact of fly feeding on dermatitis score in the host.

In *Phase 1,* each individual served as its own control as cattle were exposed to 0, 40, and 80 caged horn flies for 5 days. At the termination of the challenge skin biopsies were taken from each cage site to facilitate histology and development of a dermatitis scoring system. *Phase 1* also helped determine the optimal number of flies to be used for the next phase as determined by the impact of fly number on dermatitis score.

During *Phase 2,* cohorts of control and vaccinated cattle were created as described above. Each heifer had 4 cages sutured along their midlines on their right side. Blood was drawn before and after the vaccine was given and sent to Colorado to measure serum IgG titers. Plasma samples were submitted to the Auburn University Clinical Pathology Laboratory for the determination of total Food Animal Blood panels and CBCs. For this phase, vaccine efficacy was tested using an acute and a chronic approach. To determine the acute impact of vaccination on fly feeding behavior, 40 starved horn flies were placed within a cage and allowed to feed unabated for 25 minutes. After this exposure, the cage was removed, and midguts were dissected from the flies to allow determination of midgut bloodmeal volume. In To determine the chronic impact of vaccination upon fly health and the clinical impact of fly biting upon the host vaccinated and unvaccinated cattle were exposed to 0 and 120 caged horn flies for 3 days. The control and fly containing cages were then removed and the alive and dead flies counted and sexed to calculate fly mortality. Immediately upon cage removal, three skin biopsies were taken from within each cage site. The biopsies were then fixed and utilized for the histological scoring of dermatitis.

2.3.1 Cage attachment and design

First, cattle were administered a dose of xylazine via IV through the tail vein and lidocaine in an inverted "L" pattern. Xylazine is a sedative to reduce anxiety and lidocaine is a local anesthetic to numb the area before suturing cages on. Cattle were then shaved along the midline and ribs where horn fly cages were anticipated to be placed and sutured with nylon surgical suture. After the cage attachment, individuals were given 2 days of rest to acclimate to the placement of the enclosures. On the 3rd morning, horn flies were introduced to the cages at the exposure numbers described above. During *Phase 1* only 8 of the 9 heifers were utilized and 3 horn fly enclosures were sutured onto the left rib cage along the midline of the cattle. The enclosures were constructed from 10cm diameter schedule 40 PVC pipe with 5cm of height. This created a surface area of 81cm^2, resulting in 0.50 flies per cm^2, or 1 fly per cm^2. A 4cm hole was drilled into the center of the PVC ring to allow flies to be introduced. Following the introduction of flies into the cages, this hole was kept closed with a rubber stopper. The opening facing the dermis was constructed with window mesh in the center and a more durable plastic mesh as the outer ring for sutures to hold. The ring protruded about 4cm from the cage. Both meshes were secured to the opening of the cage with silicone caulk (GE All Purpose Silicone) and allowed to cure for at least 24 hours. Silicone was used because it is flexible, but also FDA regulated and considered food safe so as not to interfere with fly health. On the opposing end, the cage was also covered with window mesh and secured with hot glue.

During *Phase 2* slight modifications were made to improve the durability of the cages and to reduce the surface area available for horn flies to access the host dermis. The cages were reduced in size to 8cm in diameter, 4cm in height, with a surface area of 46cm^2. This resulted in a more condensed area of 3 flies per cm^2. Cages were then sewed together with fishing wire on

all 4 sides to prevent cages from coming apart. By decreasing the cage area and increasing fly numbers, the impact of fly exposure was increased.

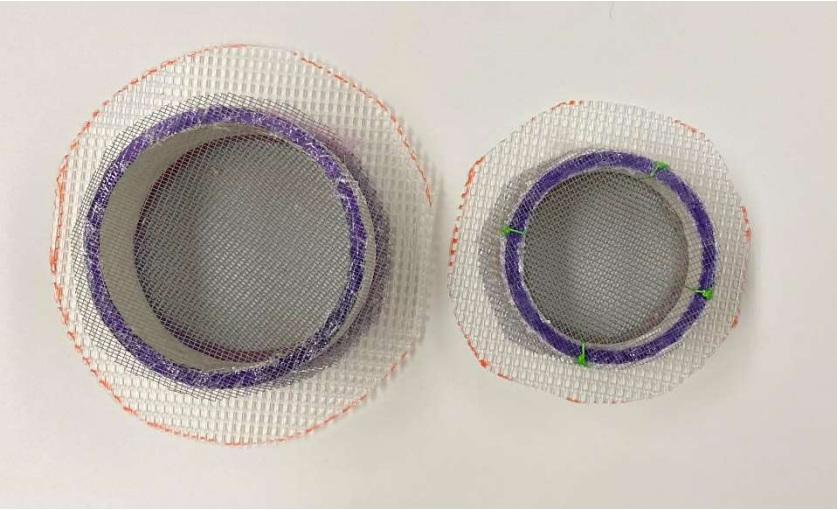

Fig. 2.1 Cage design: size alteration and modification between phase 1 (left) and phase 2 (right). Phase 1 cages were 10cm in diameter with a surface area of 324cm^2. Phase 2 cages were 8cm in diameter, 4cm in height, with a surface area of 46cm^2.

2.3.2 Collecting of wild horn flies

For these trials, horn flies were wild caught. For the chronic exposure studies, wild horn flies were collected the morning of day 0, counted, and then loaded into cages on the heifers. For the acute study conducted to assess the impact of vaccination status on blood meal volumes, horn flies were caught at 6pm and placed overnight in fly enclosures where they were given access to water. Following the overnight fast, horn flies were counted and loaded into cages for the 25-minute refeeding challenge. Sweep nets were used to collect flies from wild populations found

on resident beef cattle at the Auburn University Beef Teaching unit. Horn flies were placed into a netted cage and counted with an HB8 insect aspirator (Bugdorm, Taichung, Taiwan).

2.3.3 Vaccine and sham vaccine composition

During *Phase 2,* all 9 heifers received an injection, however only 4 of the 9 heifers were given the thrombostasin vaccine. The vaccine is composed of 3 different solubilized, full length, purified recombinant thrombostasin protein isotypes, TS8, TS9, and TS10, suspended in an equal mixture of saline buffer with the licensed ENABL adjuvant. The recombinant thrombostasins are expressed in E. coli using the pET30a expression plasmid and purified via affinity columns using the C-His6tag to a purity that is greater than 80% as judged by SDS-PAGE analysis. The recombinant proteins are lyophilized after dialysis and are readily soluble in sterile water. The vaccine is created off site by TNG Pharma and sent to us in Auburn ready to use. Antigen levels per thrombostasin protein were 100ug each per dose. In this study the vaccine was administered in 2 separate doses, the priming and boosting dose, via intramuscular injection. Each animal received 2cc of the experimental vaccine per dose intramuscular through a 14-gauge needle. The animals were initially vaccinated in October and re-vaccinated again as naive animals in May. The priming dose was given on May 26, 2023 and the boosting dose on June 23, 2023. Heifers were given a week of rest before cages were attached for experiments.

2.3.4 Quantitative comparison of blood volume in fly midguts

For this experiment horn flies were collected from a wild population via sweep netting and placed in a netted cage to be starved with a cotton ball soaked in water for over 12 hours at room temperature. The preliminary experiment took place on day 9 of the experiment, after horn flies were starved overnight on day 8. On day 9 and 10 wild horn flies were caught and placed

into netted cages and starved overnight for over 12 hours. The experiment comparing blood volume of midguts occurred on day 10 and 11 of the experiment. 2 to 4 starved horn fly midguts were dissected and collected at the start of every replicate to demonstrate a baseline for empty midguts. 40 horn flies were then counted with a hand pump aspirator and placed into cages and allotted 25 minutes to feed on either a vaccinated or unvaccinated heifers. After 25 minutes, the cage was removed and placed immediately on ice to anesthetize the flies. Female horn flies were separated, and mid guts were surgically removed. All dissections were conducted under a stereo microscope with fly bodies immersed in 1x Phosphate-buffered saline. Each dissected midgut was placed into 250 µl of Drabkin's reagent (Ricca Chemical, Arlington, TX) and homogenized with a battery-operated motorized pestle mixer (Cole-Parmer, Vernon Hills, IL). The samples were then incubated for 30 minutes upon which 200 µl of each homogenate was transferred to a 96 well plate whereby sample absorbances were determined using a spectrophotometer at 540 nm. A standard curve was constructed by assaying known quantities of fresh whole blood taken randomly from one of the heifers and mixed with EDTA to prevent coagulation. The blood was mixed with Drabkin's reagent and homogenized with a motorized pestle and the standard curve was constructed using 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2 µl, and 4 µl following the same procedure as the samples.

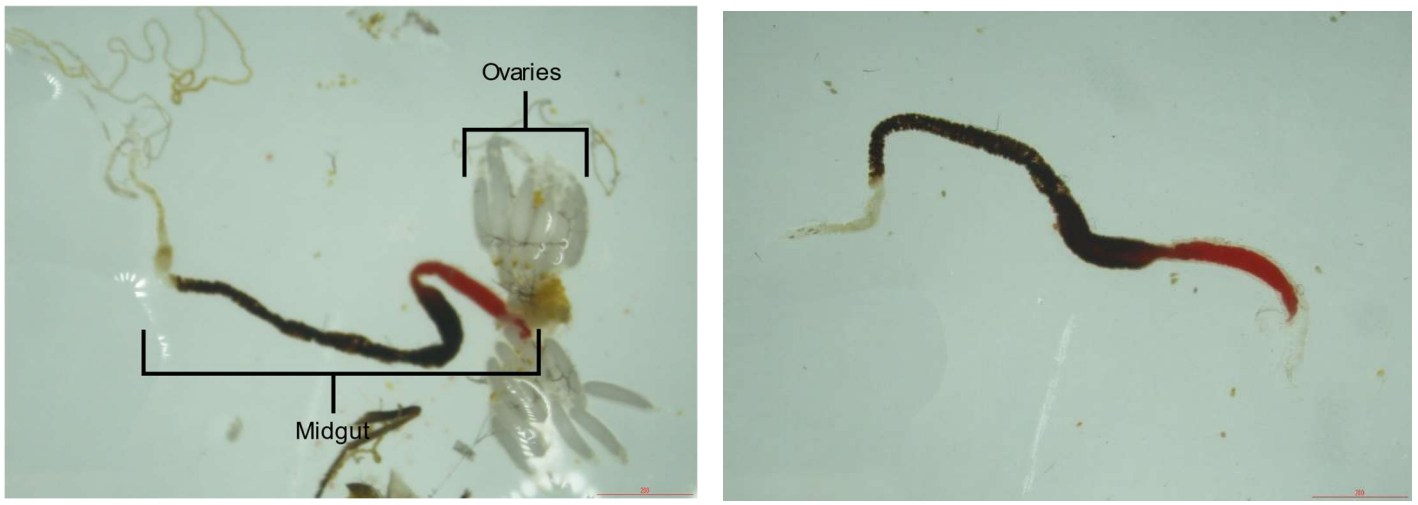

Fig. 2.2 Dissection of Midgut in female horn fly. The photo on the left depicts the ovaries, midgut, and hind gut of a female horn fly. The photo on the right depicts the bare midgut used in the blood volume challenge.

2.3.5 Analysis of mortality rate between flies feeding on vaccinated or unvaccinated cattle

In this experiment horn flies were collected from a wild population via sweep netting and promptly counted using a hand pump aspirator. On day zero, cages were sutured onto each heifer and on day two 120 horn flies were placed into cages attached to the heifers and left to feed unabated for three days. On the morning of day five, the sutures were clipped and the cages were removed to count the live and dead flies. This was done by placing the pvc cages into a netted cage and removing the top window mesh to release the live flies. Dead flies were counted, and live ones were briefly placed in a freezer to anesthetize the flies for easier counting. After live and dead horn flies were separated and placed into different bags, a cotton ball soaked in acetone was placed with the live ones to kill them. The originally live flies were then sexed under a scope.

2.3.6 Histology

Once challenge cages were removed on day five, three 6mm skin punch biopsies were collected randomly from within each cage site and placed in formalin for at least 24 hours to fix samples. Each specimen was bisected and processed for routine histopathology. One half of each punch was cut into 4 micrometer thick slides and stained with hematoxylin and eosin. Photomicrographs were taken with an image viewer software (OlyVIA; Olympus) and digital slides were processed with a research slide scanner (Slideview VS200; OI).

To determine a dermatitis scoring system, all slides were evaluated blindly by one pathologist (Dr. Rachel Neto). The pathologist utilized a semi quantitative histological analysis, which primarily focused on changes in dermal and subcutaneous inflammation through numbers of immune infiltrates such as eosinophils, lymphocytes, and plasma cells. The scoring system for dermal inflammation is represented in Table 2.1.

2.3.7 Statistical Analysis

Data were analyzed as a completely randomized block design using a mixed linear model of SAS v9.2 (SAS Institute, Inc., Cary, NC, USA) with an individual animal serving as the experimental unit (i.e., individual block) for percent viability and mid-gut blood meal volume experiments. Individual fly midgut served as the experimental unit for the initial Drabkin's experiment comparing fed, fasted, and refed flies. Values presented are group mean ± SEM, with differing superscripts within a variable denoting difference between vaccination status for live animal experiments and differences between nutritional status for the initial Drabkin's experiment., $p < 0.05$.

2.4 Results

2.4.0 Development of dermatitis scoring scale and determination of optimal horn fly exposure

To determine the appropriate horn fly exposure to induce clinical dermatitis and develop a dermatitis scale to measure in the bovine, we conducted a five-day caged fly challenge using 0, 40, and 80 flies per 4 cm in diameter cages. Biopsies collected from under the cage at the end of the challenge sectioned, stained, and blindly scored by a histologist. Confirming skin biopsy quality, all three layers of the dermis were observed in stained sections allowing a scoring system based on upon eosinophil migration and presence within the representative field of views. This resulting semi-quantitative scoring system of subcutaneous leukocytes is depicted in Table 2.1. A visual representation of these scores is depicted in Figure 2.3. Biopsy samples were then scored across 8 heifers. Dermatitis scoring was highly variable between heifers, however, in general, fly exposure associated with a change in dermatitis score when examined within animal (Figure 2.4). Upon consultation with the pathologist, it was determined that it was appropriate to increase fly exposure to induce a more definitive clinical dermatitis. This understanding led to changes in fly exposure to now reflect 120 flies per cage utilizing a cage design with a decreased surface are and total volume thus in affect increasing the fly exposure “dosing”.

Table 2.1 Scoring system for histopathology of cutaneous leukocytes.

Feature	Score	Description	Magnification
Eosinophils	0	Absent	200x
	1 - Mild	1-2 juxtaposed perivascular infiltrates	
	2 - Moderate	3-4 juxtaposed perivascular infiltrates	
	3 - Marked	≥5 juxtaposed perivascular infiltrates	
Lymphocytes and plasma cells	0	Absent	200x
	1 - Mild	1 juxtaposed perivascular infiltrates	
	2 - Moderate	2 juxtaposed perivascular infiltrates	
	3 - Marked	≥3 juxtaposed perivascular infiltrates	

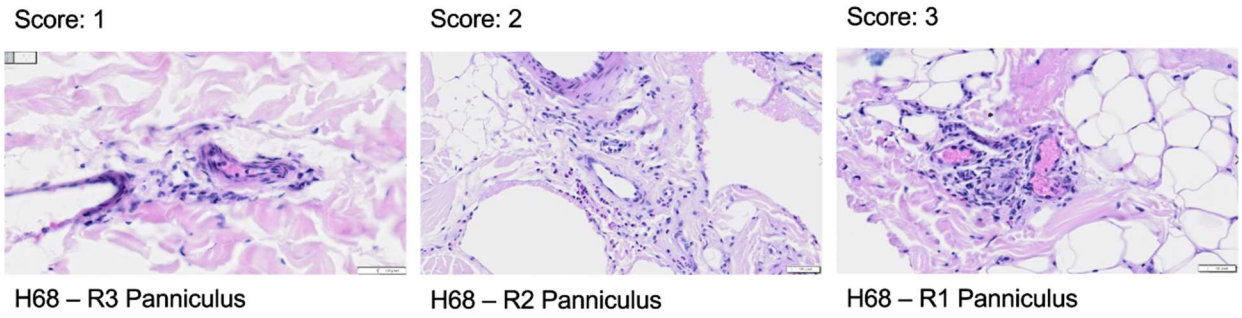

Fig. 2.3 Visual Representation of Dermatitis Scores. Dermatitis scoring was established by analyzing skin biopsies taken from the same heifer (H68) but at different locations (R1, R2, R3) in order to capture different densities of eosinophils, lymphocytes, and plasma cells within the scoring field of view. A score of 1 represents a mild dermatitis response, 2 represents a mild response, and 3 represents a marked response. These scores reflect those indicated in Table 2.1 based upon eosinophils.

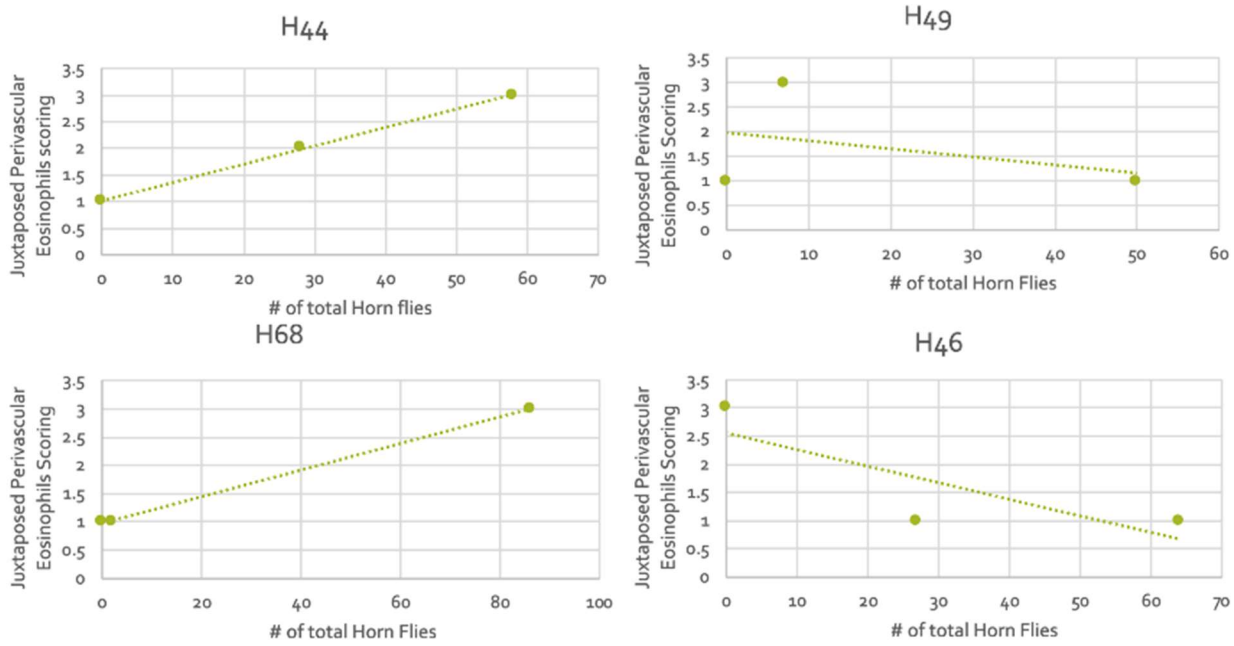

Fig. 2.4 Representation of the relationship between fly exposure and dermatitis scoring within an individual heifer. Shown are the dermatitis scores associated with 0,40, and 80 flies per 4cm diameter cage**.** Representative graphs are associated with unvaccinated heifers H44, H46, H49, and H68. The y-axis demonstrates the blind score deemed by the histologist.

2.4.1 Midgut blood volume determination

An initial experiment was conducted to evaluate our ability to detect meaningful differences in midgut blood volume using the Drabkins assay approach and to ensure our fasting duration was not deleterious to the ability of horn flies to feed robustly during cage challenges. To accomplish this aim, three groups of wild caught horn flies were compared- 1) ones caught during feeding in real time on the midlines of cattle, 2) wild caught horn flies that were fasted overnight, and 3) fasted horn flies that were allowed to refeed unabated for 25 minutes while in fly cages. As shown in Figure 2.5A, dissected midguts from wild horn flies caught during a feeding bout on cattle and immediately dissected contained an average of .44 µl of blood. fasted

for 12 hours had a reduced midgut blood volume of 86% (0.062 µl/midgut; $p < 0.05$) when compared to wild flies caught directly feeding off of cattle. By comparison, allowing fasted flies to refeed unabated within cages for 25 minutes resulted in midgut blood volumes that were 1161% higher (0.72µl/midgut) than their fasted ($p < 0.001$) and 62% higher ($p < 0.05$)th than their wild fed or fasted counterparts. The equation of the standard curve constructed to determine unknown values was y=0.3013 + 0.0014 and the R2= 0.991.

Next, to determine if vaccination status of their hosts had an impact on midgut blood meal volume in horn flies, wild caught and fasted flies were placed in challenge cages sutured to vaccinated or naïve (control) heifers. As shown in the Figure 2.5B, vaccination status significantly impacted blood meal volume within the midgut of the horn flies that were refed on vaccinated cattle displaying a blood volume that was 46% lower than blood meal volumes in midguts of horn flies that were refed on control animals ($p < 0.05$). The equation for the standard curve utilized to determine unknown values was y=.2231 + .0021 and the R2 =.9999.

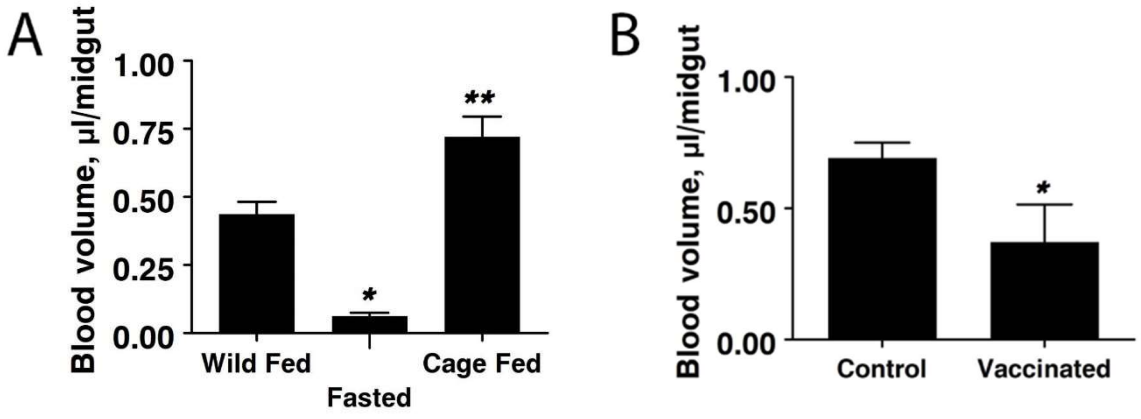

Fig. 2.5 Impact or nutritional and vaccination status on horn fly midgut blood volumes. ***Panel A*** depicts the impact average midgut blood volumes of horn flies that were 1) caught off the midlines of cattle and immediately dissected, 2) subjected to an overnight fast before midgut dissection, and 3) were fasted then refed within cages on cattle for 25 minutes. ***Panel B*** depicts the impact of vaccination status on the average midgut blood volume in wild caught flies that were fasted overnight and then allowed to refeed unabated for 25 minutes in cages on either control or vaccinated heifers. Means represent n= 15-20 midguts (***Panel A***) and n=5, Control, and n=4, vaccinated (***Panel B***) Differences in means are denoted * ($p < 0.05$) or ** ($p < 0.001$).

2.4.2 Mortality comparison between horn flies feeding on vaccinated and unvaccinated cattle.

To evaluate the efficacy of the vaccine through fly mortality, 120 wild caught horn flies were placed into challenge cages of vaccinated and unvaccinated heifers for a duration of 3 challenge days (Figure 2.6). Cages were then removed, and live/dead horn flies counted and sexed. Horn flies feeding on control animals had a 59.5% viability ± 4.6, while horn flies feeding on vaccinated had a 35% viability ± 13.3. Horn flies feeding on the unvaccinated animals (control) showed a 24% higher viability when compared to horn flies feeding on vaccinated animals ($p < 0.05$).

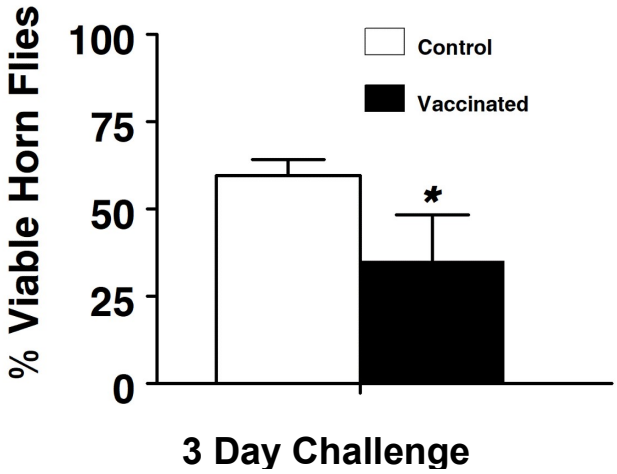

Fig 2.6 Comparison of percent viability of horn flies feeding on vaccinated and unvaccinated cattle after 3 days. Following completion of the 3 day challenge, cages were removed and numbers of live versus dead flies were counted within each cage. Values are reported as the mean number of live flies divided by total flies per cage. The means represent the average of 5 control animals and 4 vaccinated animals with differing asterisks denoting difference between variables ($p < 0.05$).

2.5.3 Dermatitis scoring of vaccinated and unvaccinated cattle exposed to horn flies

During phase 1, a dermatitis scoring system was developed to aid in scoring markers of dermatitis within bovine skin biopsies for phase 2. Each animal served as its own control which increases statistical power. with fly exposures increasing from 0, to 40, to 80 flies per cage within heifer. Although the outcome of dermatitis caused by horn fly dosage was inconsistent between cattle in phase one, increasing fly concentration tended to increase dermatitis score within each individual heifer. The results of Phase 2, comparing the dermatitis scores between vaccinated and control animals exposed to 120 horn flies for 3 days is currently still being analyzed by the pathologist.

2.5 Discussion:

Horn flies are one of the most economically important ectoparasites in the world. These hematophagous pests decrease cattle production efficiency, vector cattle diseases, and cause major financial loss for producers. These issues have both direct and indirect impacts on food production contributing to food insecurity as our population grows and demand for food continues to rise. The current management methods such as insecticides have lost potency as horn flies quickly develop resistance to such agents causing increasingly greater need for innovative solutions such as the thrombostasin vaccine to inhibit horn fly feeding (Oyarzún, Quiroz, and Birkett 2008).

Thrombostasins are currently the only known salivary proteins utilized by horn flies capable of inhibiting blood clotting in cattle (Cupp et al. 1998). Therefore, the development of a vaccine against thrombostasins may represent a new tool that allows effective control of horn fly populations. Previous experimental studies have supported the idea that vaccinating cattle against different isoforms of thrombostasins results in altered blood meal uptake by horn flies (Cupp et al. 2010). These experiments demonstrate that vaccinating cattle against various recombinant thrombostasin isoforms induces elevated IgG titers for antibodies directed against thrombostasins in cattle suggesting the strategy is a viable way to induce immunity. These previous experiments also prove that the population of horn flies has multiple isoforms of thrombostasins. This has led to the development of a novel cocktail vaccine that is made up of TS8, TS9, and TS10. By creating a cocktail vaccine against multiple isoforms of thrombostasins, experiments can be conducted to test the efficacy of the vaccine against wild populations of horn flies within Auburn, Alabama.

An effective way to measure horn fly feeding behavior is to measure the blood meal content within the midgut immediately after feeding. Presumably, blood meal volume should correlate directly with feeding behavior. By dissecting out the midguts of anesthetized horn flies after feeding, a measurement of hemoglobin content can be determined with the use of Drabkin's reagent (Cupp et al. 2010). The Drabkins method can measure all haemoglobin forms and consists of a cocktail of potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate which oxidizes haemoglobin to cyanmethemoglobin which is easily estimated at 530nm (Balasubramaniam, P., and A. Malathi, 1992). Midgut blood volume can be considered a direct measurement of fly feeding behavior because there is an increase in midgut blood volume after feeding. This can be seen in Figure 2.5A where fasted horn flies demonstrate the lowest amount of midgut blood volume (0.062 µl) and fasted flies fed for 25 minutes demonstrate an 11-fold increase in midgut volume (0.72 µl). These midgut blood volumes are consistent with literature even when collection approach differed. Field collected horn flies of mixed age and sex held approximately 1.5mg of blood per meal on average when measuring blood volume via an excreta-based strategy, which converts to approximately 1.5 µl of blood (Kuramochi and Nishijima 1980; Brewer et al. 2021). Previously, Drabkin's reagent was utilized to compare blood uptake of horn flies fed on cattle vaccinated with different isoforms of thrombostasins for 25 minutes and midgut volumes ranged from 0.3 µl to 1.1 µl (Cupp et al. 2010). These previous measurements of midgut blood volume are consistent with the volumes collected in this study. Fasting did not negatively impede health of the flies because they demonstrated a robust feeding behavior when placed within cages which provided unabated access to the host dermis as seen in Figure 2.5A. Our blood meal volumes generally agreed with those reported in the literature being lower than volumes derived from exreta-based methods but consistent with those estimated using

the Drabkin's approach. Interestingly, fasted flies that were allowed to refeed for 25 minutes showed higher midgut blood volume when compared to midguts of wild caught flies. This again suggests that fasting did not impact horn fly feeding behavior and our challenge design mimics robust fly biting.

To test the efficacy of the vaccine on horn fly feeding, a comparison of midgut blood volume was conducted between starved horn flies that were allowed to refeed on either vaccinated or unvaccinated cattle. Since midguts were immediately dissected and blood volume quantified, the data should correlate directly with horn fly feeding behavior. Horn flies feeding on vaccinated cattle were unable to feed as effectively as those that were placed on unvaccinated animals as evidenced by significantly lower blood meal volumes being present in midguts dissected from flies harvested from the vaccinated hosts. This supports the hypothesis that vaccinating against thrombostasins can interrupt horn fly feeding.

Consistent with the apparent interruption of fly feeding, caged flies exposed to vaccinated cattle for three days displayed greater mortality than their counterparts allowed to feed on control animals over this same duration. This suggests that interrupting fly feeding may induce physiological stresses such as negative energy balance which ultimately may impact fly longevity. The mortality test is beneficial because it provides a binary and definitive outcome concerning the impact of vaccination status upon the fly. One limitation to this experiment was that given the need to utilize wild caught flies as the fly source, fly samples represented cohorts with unknown mixed age and sex which could potentially impact both blood meal volumes and viability. However, a wild population may be the best model to use when testing a novel vaccine for commercial use given wild caught flies most closely model true production conditions in which the vaccine would be used. Regardless, the observed decrease in viability displayed by

horn flies exposed to vaccinated cattle is consistent with the hypothesis that vaccinating cattle against thrombostasins can alter the horn fly life cycle.

The mortality test is an indirect measurement of horn fly feeding behavior that at best may correlate differences in feeding behavior to survivability as a marker of the potential impact of such differences on horn fly physiology. Horn flies that are unable to feed efficiently are more likely to have lower fecundity and survivability than those able to feed unabated. Indeed, such a relationship was observed in the present study as horn flies exposed to vaccinated cattle exhibited higher percentages of mortality than those that fed on unvaccinated cattle. This suggests that the vaccine inhibits feeding behavior enough to cause downstream physiological adaptations that are stressful enough to impact longevity of the horn fly. Such data provide tangible evidence to support licensing of the experimental vaccine for commercial use. (Clinical aspect/ dermatitis has yet to be scored)

In summary, these data indicate that vaccinating cattle against thrombostasins directly impacts the feeding behavior and physiology of horn flies. These data support the model that antibodies produced by cattle after exposure to the vaccine prevents horn flies from feeding as effectively as they would on unvaccinated cattle; thereby leading to lower amounts of blood meal taken in the short term and higher rates of mortality in the long term. These data support our hypothesis that vaccinating cattle against thrombostasins is an efficacious strategy to control horn fly populations and provides positive evidence in support of its licensing for commercial use within the cattle industry.

References:

Brewer, Gary J., Dave J. Boxler, Luísa N. Domingues, Rebecca T. Trout Fryxell, Chris Holderman, Kelly M. Loftin, Erika Machtinger, Brandon Smythe, Justin L. Talley, and Wes Watson. 2021. "Horn Fly (Diptera: Muscidae)—Biology, Management, and Future Research Directions." Journal of Integrated Pest Management 12 (1). https://doi.org/10.1093/jipm/pmab019.

Burkett-Cadena, Nathan D., Erik M. Blosser, and Lawrence E. Reeves. 2022. "Key to the Adult Females of Species of Culex Subgenus Melanoconion in Florida, USA." Journal of the American Mosquito Control Association 38 (3): 130–40.

Byford, R. L., M. E. Craig, S. M. DeRouen, M. D. Kimball, D. G. Morrison, W. E. Wyatt, and L. D. Foil. 1999. "Influence of Permethrin, Diazinon and Ivermectin Treatments on Insecticide Resistance in the Horn Fly (Diptera: Muscidae)." International Journal for Parasitology 29 (1): 125–35; discussion 137–38.

Cleaveland, S., M. K. Laurenson, and L. H. Taylor. 2001. "Diseases of Humans and Their Domestic Mammals: Pathogen Characteristics, Host Range and the Risk of Emergence." Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 356 (1411): 991–99.

Cupp, E. W., M. S. Cupp, J. M. Ribeiro, and S. E. Kunz. 1998. "Blood-Feeding Strategy of Haematobia Irritans (Diptera: Muscidae)." Journal of Medical Entomology 35 (4): 591–95.

Cupp, Mary S., Eddie W. Cupp, Christine Navarre, Dunhua Zhang, Xin Yue, Latora Todd, and Victor Panangala. 2010. "Salivary Gland Thrombostasin Isoforms Differentially Regulate

Blood Uptake of Horn Flies Fed on Control- and Thrombostasin-Vaccinated Cattle." Journal of Medical Entomology 47 (4): 610–17.

Cupp, Mary S., E. W. Cupp, Christine Navarre, Nancy Wisnewski, Kevin S. Brandt, Gary M. Silver, Dunhua Zhang, and Victor Panangala. 2004. "Evaluation of a Recombinant Salivary Gland Protein (thrombostasin) as a Vaccine Candidate to Disrupt Blood-Feeding by Horn Flies." Vaccine 22 (17-18): 2285–97.

Cupp, M. S., D. H. Zhang, and E. W. Cupp. 2000. "Horn Fly (Diptera: Muscidae) Saliva Targets Thrombin Action in Hemostasis." Journal of Medical Entomology 37 (3): 416–21.

Ellwanger, Joel Henrique, and José Artur Bogo Chies. 2021. "Zoonotic Spillover: Understanding Basic Aspects for Better Prevention." Genetics and Molecular Biology 44 (1 Suppl 1): e20200355.

Gordon, D. V., W. D. Haufe, and K. K. Klein. 2008. "Determination of Economic Thresholds for Horn Fly Control in Western Canada: A Farm Level Simulation Approach." Canadian Journal of Agricultural Economics 32 (2): 399–421.

Grisi, Laerte, Romário Cerqueira Leite, João Ricardo de Souza Martins, Antonio Thadeu Medeiros de Barros, Renato Andreotti, Paulo Henrique Duarte Cançado, Adalberto Angel Pérez de León, Jairo Barros Pereira, and Humberto Silva Villela. 2014. "Reassessment of the Potential Economic Impact of Cattle Parasites in Brazil." Revista Brasileira de Parasitologia Veterinaria = Brazilian Journal of Veterinary Parasitology: Orgao Oficial Do Colegio Brasileiro de Parasitologia Veterinaria 23 (2): 150–56.

Guglielmone, A. A., E. Gimeno, J. Idiart, W. F. Fisher, M. M. Volpogni, O. Quaino, O. S. Anziani, S. G. Flores, and O. Warnke. 1999. "Skin Lesions and Cattle Hide Damage from Haematobia Irritans Infestations." Medical and Veterinary Entomology 13 (3): 324–29.

Harris, R. L., J. A. Miller, and E. D. Frazar. 1974. “Horn Flies and Stable Flies: Feeding Activity.” Annals of the Entomological Society of America 67 (6): 891–94.

Koh, Cassandra, Lionel Frangeul, Hervé Blanc, Carine Ngoagouni, Sébastien Boyer, Philippe Dussart, Nina Grau, Romain Girod, Jean-Bernard Duchemin, and Maria-Carla Saleh. 2023. “Ribosomal RNA (rRNA) Sequences from 33 Globally Distributed Mosquito Species for Improved Metagenomics and Species Identification.” eLife 12 (January). https://doi.org/10.7554/eLife.82762.

Kuramochi, Katsuhisa. 1985. “Studies on the Reproductive Biology of the Horn Fly, Haematobia Irritans (L.) (Diptera: Muscidae) II. Effect of Temperature on Follicle Development and Blood Meal Volume of Laboratory-Reared Flies.” Applied Entomology Zoology. https://www.jstage.jst.go.jp/article/aez1966/20/3/20_3_264/_article/-char/ja/.

Kuramochi, Katsuhisa, and Yutaka Nishijima. 1980. “Measurement of the Meal Size of the Horn Fly, Haematobia Irritans (L.) (Diptera: Muscidae), by the Use of Amaranth.” Applied Entomology Zoology. https://www.jstage.jst.go.jp/article/aez1966/15/3/15_3_262/_pdf/-char/en.

Lancaster, J. L., Jr, and M. V. Meisch. 1986. “Arthropods in Livestock and Poultry Production.” In Arthropods in Livestock and Poultry Production. Ellis Horwood.

Main, Bradley J., Matteo Marcantonio, J. Spencer Johnston, Jason L. Rasgon, C. Titus Brown, and Christopher M. Barker. 2021. “Whole-Genome Assembly of Culex Tarsalis.” G3 11 (2). https://doi.org/10.1093/g3journal/jkaa063.

Melo, Lídio Ricardo Bezerra, Márcia Alves Medeiros, Lucas Alencar Fernandes Beserra, Antônio Thadeu Medeiros Barros, Franklin Riet-Correa, Sérgio Santos Azevedo, and Vinícius Longo Ribeiro Vilela. 2020. “Development and Number of Generations of

Haematobia Irritans (Diptera: Muscidae) in Bovine Fecal Masses in the Semiarid Region of Brazil." Veterinary Parasitology, Regional Studies and Reports 20 (April): 100411.

Narayan, Edward, and Naureen Rana. 2023. "Human-Wildlife Interaction: Past, Present, and Future." BMC Zoology 8 (1): 5.

Oyarzún, M. P., A. Quiroz, and M. A. Birkett. 2008. "Insecticide Resistance in the Horn Fly: Alternative Control Strategies." Medical and Veterinary Entomology 22 (3): 188–202.

Showler, Allan T., Weste L. A. Osbrink, and Kimberly H. Lohmeyer. 2014. "Horn Fly, Haematobia Irritans Irritans (L.), Overwintering." International Journal of Tropical Insect Science 6 (May). https://doi.org/10.4137/IJIS.S15246.

Williams, Martin R., and Harry M. Savage. 2011. "Development of Multiplexed Species Specific Polymerase Chain Reaction Assays for Identification of the Culex (Melanoconion) Species (Diptera: Culicidae) of the Southeastern United States Based on rDNA." Journal of Medical Entomology 48 (5): 961–66.

Woolhouse, Mark E. J., and Sonya Gowtage-Sequeria. 2005. "Host Range and Emerging and Reemerging Pathogens." Emerging Infectious Diseases 11 (12): 1842–47.

Appendix 1

Viral Profile of *Culex erraticus*

3.1 Introduction

Nearly 75% of emerging diseases are of zoonotic origin (Woolhouse and Gowtage-Sequeria 2005) and 26% of all human pathogens are capable of infecting domestic and wild animals (Cleaveland, Laurenson, and Taylor 2001). Zoonotic diseases often develop from spillover events when a pathogen becomes capable of being transmitted from a wild host to a human (Ellwanger and Chies 2021). These diseases pose a huge threat to global health security and the emergence of these pathogens are only expected to increase as urbanization and human population continues to grow leading to an increase of interactions with wildlife (Narayan and Rana 2023).

Zoonotic disease transmission can also be facilitated by hematophagous ectoparasites such as mosquitoes (CDC 2023). Certain species of mosquitoes prefer more urban areas, whereas more uncommon species prefer tall grasses and forests. This is a concern because as humans encroach further into wild habitats, they run a higher risk of interacting with mosquitoes that have already fed on other animals and picked up a zoonotic disease. All mosquitoes are commonly found near stagnant water sources due to aquatic larval and pupal life stages (Nordgulen, Burkett-Cadena, and Mathias, n.d.).

Due to these circumstances, a prime location to investigate mosquito viruses would be at the E.W. Shell Fisheries in Auburn, AL. This is because E.W. Shell Fisheries is composed of 1600 acres of stock ponds, which provide ample habitat for mosquitoes and waterfowl. It is also located south of Kreher preserve, which provides wilder habitat and higher opportunity for

mosquito and wildlife interaction. The fisheries are commonly frequented by students and researchers for maintenance and care of aquaculture, which overlap with wildlife and mosquitoes allowing for opportunities for zoonotic diseases to be transmitted.

This study focuses on a particularly common mosquito species known as *Culex erraticus.* This species was one of the most common captured at the fisheries and is known to feed on avian hosts early in the season before transitioning to mammals (Oliveira et al. 2011). This species is also capable of transmitting zoonotic diseases including West Nile Virus and Eastern Equine Encephalitis (Bingham et al. 2016; E. W. Cupp et al. 2004). There is a chance that these mosquitoes can be harboring other viruses that may be detrimental to people or livestock. By using Next Generation Sequencing, a viral profile of this mosquito species can be uncovered and studied before it makes the jump to humans.

3.2 Materials and Methods

3.2.0 Study Site

Mosquitoes utilized for this study were trapped at Auburn University, E.W. Shell Fisheries center located in Auburn, AL. USA. The E.W. Shell Fisheries center is a 1600-acre research station consisting of infrastructure and natural habitat. Man-made ponds facilitate studies utilizing diverse species of catfish and trout. The fisheries also provide a habitat for a variety of wildlife including Bald Eagles, Belted Kingfishers, blue herons, wading birds, songbirds, reptiles, and amphibians. The fisheries are located on AL- 147, Auburn, Alabama (Coordinates) south of the Kreher Preserve & Nature Center and north of Auburn campus. This unique location of the fisheries allows it to serve as a novel model representing the interface

between a more urban environment to the south and a more natural environment to the north. This location allows for a more biodiverse ecosystem to study zoonosis.

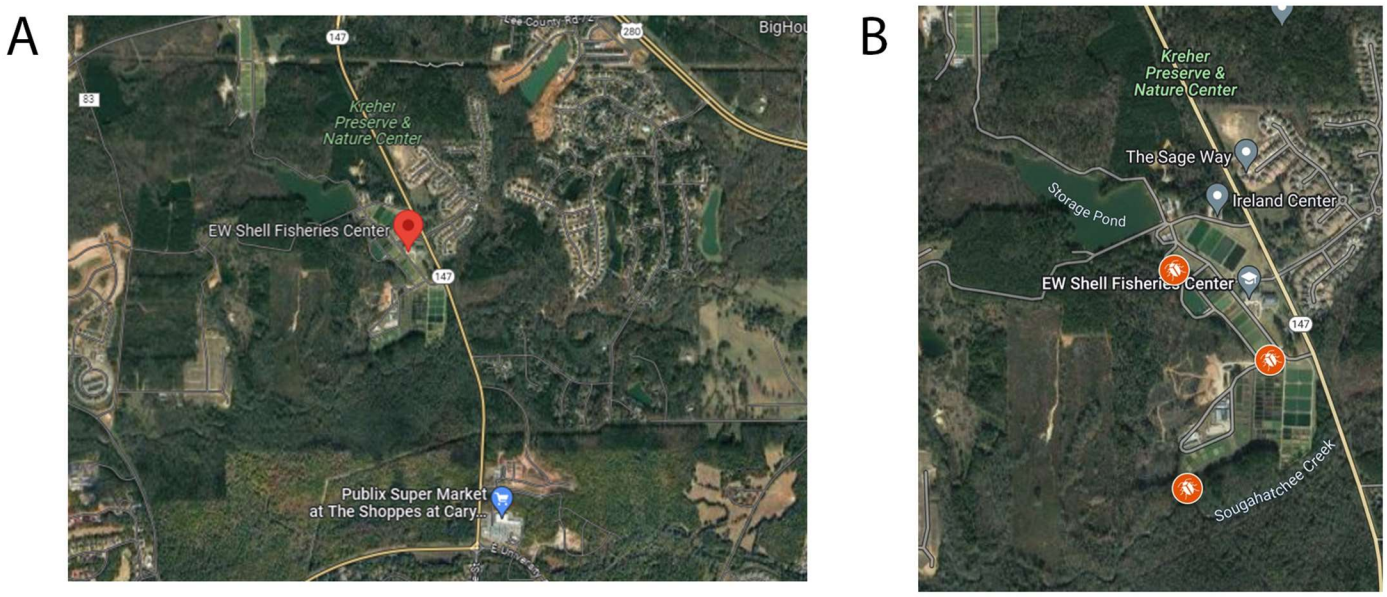

Fig. 3.1 Mosquito Trap Locations. Figure 3.1A depicts the location of E.W. Shell Fisheries in Auburn, AL. It is positioned between the Kreher Preserve and more urban parts of town such as Publix Super Market. Figure 3.1B depicts the locations within E.W. Shell Fisheries at which the CDC miniature light traps were placed.

3.2.1 Mosquito Collection and Taxonomic Identification

Live adult mosquitoes were collected with the use of Centers of Disease Control miniature light trap Model 512 (John W. Hock) baited with CO2 emitted from ~2lbs of dry ice per trap. Traps were set at several sites within the E.W. Shell Fisheries in Auburn, Alabama and demonstrated in Figure 3.1B. These sites were located either along the edge of the fisheries and forests or at recently drained ponds to capture more diverse populations of mosquitoes. Traps were set overnight from 4pm until 10am the next morning and the collection period lasted

between late August through the end of October 2022. The live adult mosquitoes were then transported to the laboratory and killed by incubation at -20 degrees Celsius for 10 minutes. Mosquitoes were then sexed and morphologically classified by species with the use of a stereomicroscope. Each species and gender were grouped into pools with a maximum of 20 adult mosquitoes per pool. Each pool was submerged in 100% ethanol and kept in -20 degrees Celsius until nucleic acid extraction.

Table 3.2 Mosquito Collection at the Fisheries.

Date Collected	Collection #	Collection Site	Species	Female/ Male	Total
8/23/2022	AA	Fisheries: Creek	Culex Erraticus	F	24
8/31/2022	AC	Fisheries: Creek	Culex Erraticus	F	12
8/31/2022	AC	Fisheries: Creek	Anopheles Crucians	F	1
9/7/2022	AF	Fisheries: Creek	Culex Erraticus	F	8
9/7/2022	AG	Fisheries: Pond	Culex Erraticus	F	5
9/7/2022	AG	Fisheries: Pond	Psorophora	F	5
9/21/2022	AI	Fisheries: Creek	Culex Erraticus	F	80
9/21/2022	AJ	Fisheries: Right pond	Culex Erraticus	F	4
10/12/2022	AN	Fisheries: Creek	Culex Erraticus	F	20
10/26/2022	AP	Fisheries: Creek	Culex Erraticus	F	80
10/26/2022	AP	Fisheries: Creek	Psorophora	F	1

This table depicts the mosquito collections taken from E.W. Shell Fisheries between late August to late October in 2022.

3.2.2 Viral RNA Extraction

Female *Culex erraticus* mosquitoes were removed from ethanol and allowed to dry under a laminar flow hood for 10 minutes. Individuals were then pooled into four different groups ranging from 15 to 17 mosquitoes and placed into 1.5ml micrcentrifuge tubes. Samples were homogenized by pestle in liquid nitrogen and RNA was extracted from each group with 1ml of Trizol (Invitrogen, Waltham, MA) according to manufacturer's instructions. Modifications to

manufacturer's instructions were implemented to increase the yield and quality of RNA. These steps included repeating the chloroform extraction twice, conducting salt precipitation with 100% Isopropanol and Sodium Acetate (pH 5.2), and 2 additional cold 70% ethanol washes.

Total RNA concentration was quantified by nanodrop (Thermo Scientific, Waltham, MA) and ranged from 1600 ng/ul to 1800 ng/ul. The 260/280 ranged from 2.01 to 2.05 and 260/230 ranged from 1.88 to 1.92. RNA samples from the four different collections were pooled to create 1 sample for sequencing. Their concentrations were normalized, so that each sample contributed equally within the final pooled sample. Concentrations can be seen in Table 3.4.

Table 3.2 Mosquito used for RNA Extraction.

Date Collected	Collection #	Collection Site	Species	Female/ Male	Total
8/23/2022	AA	Fisheries: Creek	Culex Erraticus	F	24
8/31/2022	AC	Fisheries: Creek	Culex Erraticus	F	12
8/31/2022	AC	Fisheries: Creek	Anopheles Crucians	F	1
9/7/2022	AF	Fisheries: Creek	Culex Erraticus	F	8
9/7/2022	AG	Fisheries: Pond	Culex Erraticus	F	5
9/7/2022	AG	Fisheries: Pond	Psorophora	F	5
9/21/2022	AI	Fisheries: Creek	Culex Erraticus	F	80
9/21/2022	AJ	Fisheries: Right pond	Culex Erraticus	F	4
10/12/2022	AN	Fisheries: Creek	Culex Erraticus	F	20
10/26/2022	AP	Fisheries: Creek	Culex Erraticus	F	80
10/26/2022	AP	Fisheries: Creek	Psorophora	F	1

This table highlights the 4 groups of collections in which the mosquitoes were taken for RNA extraction.

Table 3.3 *Culex erraticus* Extraction and Pooling.

Used for RNA Seq					
	Mosquito species	Number of indv.	Quality (260/280)	Quality (260/230)	ng/uL
Extraction 9	Culex Erraticus	15	2.05	1.88	1743.6
Extraction 10	Culex Erraticus	16	2.03	1.87	1694.6
Extraction 11	Culex Erraticus	17	2.04	1.92	1801.6
Extraction 12	Culex Erraticus	17	2.01	1.88	1564.7
Pool 1	**Amount used**				
Extraction 9	18 ul				
Extraction 10	18.5 ul				
Extraction 11	17.4 ul				
Extraction 12	20 ul				
Total:	73.9	69 ul sent to SeqCenter			
Pool 2	**Amount used**				
Extraction 9	8.07 ul				
Extraction 10	8.31 ul				
Extraction 11	7.81 ul				
Extraction 12	9 ul				
Total:	33.19 ul	15 ul sent to SeqCenter			

This table depicts the number of individual mosquitoes used for each of the 4 extractions. It also shows the quality (260/280), (260/230), and the concentration of each extraction. A total of 2 samples were sent to SeqCenter for RNASeq using the same pooling strategy where equal amounts of nucleic acid were pulled from each sample. The first sample sent to SeqCenter was 69ul and the second was 15ul after the extractions were pooled.

3.2.3 Ribosomal RNA Depletion and Sequencing

The total RNA sample was sent to SeqCenter (Pittsburgh, Pennsylvania) and prepared for RNA Seq through their traditional pipeline. The sample was treated with RNAse free DNase (Invitrogen, Waltham, MA) and library prepped using an Illumina Stranded Total RNA Prep

Ligation with Ribo-Zero Plus kit and 10bp unique dual indices (UDI) (Illumina, San Diego, CA). Custom probes were designed to deplete host (*Culex erraticus)* ribosomal RNA. These probes were based upon 5.8s, 28s, and 18s rRNA sequences. The 5.8s of *Culex erraticus* was previously sequenced (Williams and Savage 2011) and used as a reference for the custom probes. However, the 28s and 18s of *Culex erraticus* have not yet been sequenced. References from *Culex sp.* within the same subgenus, Melanoconion (Burkett-Cadena et al. 2022), were compared through BLAST and determined to be the most similar to *Culex erraticus*. This resulted in the use of 28s and 18s sequences from a *Culex (Melanoconion) sp.* found in French Guinea (Koh et al. 2023). Sequencing was performed on a NovaSeq X Plus (Illumina, San Diego, CA). This produced paired end 150bp reads. Demultiplexing, quality control, and adapter trimming was performed with bcl-convert (v4.1.5) through SeqCenter.

3.2.4 Bioinformatics

To first determine the quality of the reads we used FastQC. Based on the information provided by FastQC we trimmed and removed low quality RNA with BBMAP. Host RNA is then removed by aligning to a reference genome of *Culex tarsalis* mosquito using Kneaddata (Main et. al 2021). Bacterial, fungal, and remaining pathogens were then taxonomically identified and separated by KRAKEN 2.

3.3 Results & Discussion

3.3.0 RNA Extraction

The appropriate number of mosquitoes to use and the most efficient method in extracting RNA for sequencing was decided through a single replicate of tests. These tests were conducted with various numbers of mosquitoes consisting of 10, 20, and 50 individuals per extraction. The

quality (260/280) of the extractions ranged from 1.46 to 1.81 with concentrations between 790 ng/ ul to 2070 ng/ ul. These readings were taken with a nanodrop and it was determined that nucleic acid concentration is dependent on the number of individuals used for each extraction However, quality remained relatively the same and was dependent on skill. Due to the limited number of mosquito samples collected, it was decided that 20 mosquitoes per extraction would be the best. By using this number, it would allow for more replicates for extraction.

Extractions were also completed with Trizol because the Quick-RNA Tissue/Insect Kit (Zymo Research, Irvine, California) did not perform well with *Culex erraticus* and it produced lower concentrations. The kit was able to extract RNA from 6 *Aedes aegypti* at a time and produce good quality results (260/280) was 2.11, (260/230) was 2.07, however, concentration was significantly lower at 116 ng/ul. Lower concentration means that not all nucleic acids were captured in the columns and viruses may have been removed in this process, which may create inaccuracies and bias when determining the viral profile of *Culex erraticus.* The columns in this kit were also not compatible with *Culex erraticus* and RNA could not be efficiently extracted. For these reasons Trizol was used for extractions.

The original protocol for Trizol produced good RNA quality (260/280) that ranged from 1.9 to 2.1, however the (260/230) measured 0.9 to 1.2. These low numbers suggest organic contaminants which could interfere with reverse transcription when performing RNA Seq. To combat this, the chloroform wash step was separated into 2 separate steps and a salt precipitation was conducted with 100% Isopropanol and 3MSodium Acetate (pH 5.2). To ensure removal of organic contaminants 2 additional cold 70% ethanol washes were performed, and precipitates were allowed to dry for up to 45 minutes. This resulted in RNA measuring around 1.8 to 1.9 at (260/230), which is sufficient for RNASeq.

Table 3.4 Testing RNA Extraction Methods.

Test 1: Optimal Number of Mosquitoes			
Sample:	**Quality (260/280)**	**Concentration (ng/uL)**	
10 Mosq	1.46	786.9	
20 Mosq	1.50	912.8	
50 Mosq	1.81	2069.8	
Test 2: Trizol vs Quick RNA Insect Tissue Kit			
Sample:	**Quality (260/280)**	**Concentration (ng/uL)**	**Quality (260/230)**
Trizol (Aedes)	2.01	4953.6	1.4
Quick RNA (Aedes)	2.11	115.7	2.07
Trizol (Culex)	2.02	2989.4	1.03
Quick RNA (Culex)	N/a	N/a	N/a
Test 3: Trizol Methods with *Aedes aegypti*			
Sample:	**Quality (260/280)**	**Concentration (ng/uL)**	**Quality (260/230)**
Original protocol	1.76	3430 ng/uL	0.51
Original protocol with additional Choloroform step	1.71	2068.4 ng/uL	0.29
New protocol	2.05	2446.4 ng/uL	1.35
New protocol with additional Choloroform step	2.06	2288.1 ng/uL	1.63

The first test in this table depicts the quality and concentration of nucleic acid extracted from 10, 20, and 50 *Aedes aegypti* mosquitoes. This was completed as a single replicate to determine the optimal number of individual mosquitoes to use per extraction. Test 2 compared different RNA extraction methods through Trizol and Quick RNA kit from Zymo. Although the kit was able to reduce contaminants it also significantly dropped concentration of nucleic acid and was not compatible with *Culex erraticus.* Test 3 compared 4 different methods of RNA extraction to optimize concentration and also reduce organic contamination. The new protocol includes a precipitation step with 3M sodium acetate (pH 5.2) and 2 additional 70% ethanol wash steps. The new protocol with additional chloroform step produced the highest quality RNA for RNASeq.

References

Bingham, Andrea M., Nathan D. Burkett-Cadena, Hassan K. Hassan, and Thomas R. Unnasch. 2016. "Vector Competence and Capacity of Culex Erraticus (Diptera: Culicidae) for Eastern Equine Encephalitis Virus in the Southeastern United States." Journal of Medical Entomology 53 (2): 473–76.

CDC. 2022. "Life Cycle of Culex Species Mosquitoes." Centers for Disease Control and Prevention. July 12, 2022. https://www.cdc.gov/mosquitoes/about/life-cycles/culex.html.

Cleaveland, S., M. K. Laurenson, and L. H. Taylor. 2001. "Diseases of Humans and Their Domestic Mammals: Pathogen Characteristics, Host Range and the Risk of Emergence." Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 356 (1411): 991–99.

Colón-González, Felipe J., Maquins Odhiambo Sewe, Adrian M. Tompkins, Henrik Sjödin, Alejandro Casallas, Joacim Rocklöv, Cyril Caminade, and Rachel Lowe. 2021. "Projecting the Risk of Mosquito-Borne Diseases in a Warmer and More Populated World: A Multi-Model, Multi-Scenario Intercomparison Modelling Study." The Lancet. Planetary Health 5 (7): e404–14.

Cupp, E. W., K. J. Tennessen, W. K. Oldland, H. K. Hassan, G. E. Hill, C. R. Katholi, and T. R. Unnasch. 2004. "Mosquito and Arbovirus Activity During 1997-2002 in a Wetland in Northeastern Mississippi." Journal of Medical Entomology 41 (3): 495–501.

Ellwanger, Joel Henrique, and José Artur Bogo Chies. 2021. "Zoonotic Spillover: Understanding Basic Aspects for Better Prevention." Genetics and Molecular Biology 44 (1 Suppl 1): e20200355.

Main, Bradley J., Matteo Marcantonio, J. Spencer Johnston, Jason L. Rasgon, C. Titus Brown, and Christopher M. Barker. 2021. “Whole-Genome Assembly of Culex Tarsalis.” G3 11 (2). https://doi.org/10.1093/g3journal/jkaa063.

Narayan, Edward, and Naureen Rana. 2023. “Human-Wildlife Interaction: Past, Present, and Future.” BMC Zoology 8 (1): 5.

Nordgulen, Mary, Nathan Burkett-Cadena, and Derrick Mathias. 4/2022. “A Mosquito Culex Erraticus (Dyar and Knab, 1906) (Insecta: Diptera: Culicidae: Culicini)1.” https://doi.org/10.32473/edis-IN1357-2022.

Oliveira, Ana, Charles R. Katholi, Nathan Burkett-Cadena, Hassan K. Hassan, Sibylle Kristensen, and Thomas R. Unnasch. 2011. “Temporal Analysis of Feeding Patterns of Culex Erraticus in Central Alabama.” Vector Borne and Zoonotic Diseases 11 (4): 413–21.

Reiter, P. 2001. “Climate Change and Mosquito-Borne Disease.” Environmental Health Perspectives 109 Suppl 1 (Suppl 1): 141–61.

Woolhouse, Mark E. J., and Sonya Gowtage-Sequeria. 2005. “Host Range and Emerging and Reemerging Pathogens.” Emerging Infectious Diseases 11 (12): 1842–47.

“Zoonotic Diseases.” 2023. May 10, 2023. https://www.cdc.gov/onehealth/basics/zoonotic-diseases.html.