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The Role of Adipokinetic Hormone in Female Aedes aegypti Mosquito Bloodmeal Digestion

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ABSTRACT: Expanding urbanization has extended the reach of Aedes aegypti mosquitoes, enabling bloodfeeding females to transmit pathogens, such as yellow fever, dengue fever, chikungunya, Zika fever, and others, to millions of people each year. Various tools, such as insecticides and release of Wolbachia-infected mosquitoes, among others, have been implemented to decrease the population of mosquitoes; these tools have, however, not been fully effective. In an effort to limit the spread of pathogens, this study focuses on understanding the reproductive biology of the seminal fluid proteins (SFPs) of Aedes aegypti. SFPs, which are received by the female mosquitoes during mating, are known to influence their physiology and behavior. Of all the SFPs, adipokinetic hormone (AKH), which is implicated in the metabolism of energy, stands out the most as it was recently discovered in the seminal fluid of insects. Previous studies show that AKH signaling increases the activity of digestive enzymes and the rate of digestion in flesh flies and firebugs. This study thus served to investigate if AKH plays a role in female Ae. aegypti bloodmeal digestion. The main objective of this project was to investigate if AKH increases the activity of digestion in female mosquitoes. To be able to accomplish this goal, a proper methodology for measuring and analyzing bloodmeal digestion was first investigated. Analysis of the SDS-PAGE of the samples of bloodmeal extracted from the midgut of mosquitoes from various treatment groups at different time points showed that the intensity of the 10kD band at 12 and 20hrs post-feeding was a good indicator of digestion rate. The effects of SFPs on the digestion rate were confirmed. The results from this study show that AKH may not be implicated in bloodmeal digestion. This observation brings us a step closer to understanding the full mechanism and effect of SFPs in mated female Aedes mosquitoes, as they are yet to be fully elucidated.

I. INTRODUCTION

Mosquitoes as a Vector

Aedes aegyptiis a container-breeding mosquito usually dispersed in the tropical and subtropical regions of the world and is a common vector of yellow fever, dengue fever, chikungunya, and Zika fever, among other diseases (Gubler, 2002). The pathogens of these diseases are transmitted to the host during mosquito blood feeding (Figure 1) (Chamberlain and Sudia, 1961; Romoser et al., 2004; Wu et al., 2019). Initially, the mosquito becomes a carrier of these pathogens when it bloodfeeds on an infected host. The pathogen then replicates in epithelial tissues in the midgut before moving to the salivary gland and neural systems in the mosquitoes (Chamberlain and Sudia, 1961; Romoser et al., 2004; Wu et al., 2019). During the second bloodfeeding of the vector, the viral capsids of pathogens, now in the mosquito's saliva, move directly into the body of the host via the mosquito's proboscis(Clements, 1992). The virus then undergoes its life cycle, causing illness in the host (Romoser et al., 2004). There is an escalation in the epidemics of these mosquito-transmitted diseases, puttingmillions of people around the world at risk (Shragai et al., 2017). Aedes aegyptimosquitoes, thus, serve as a threat to the human population.

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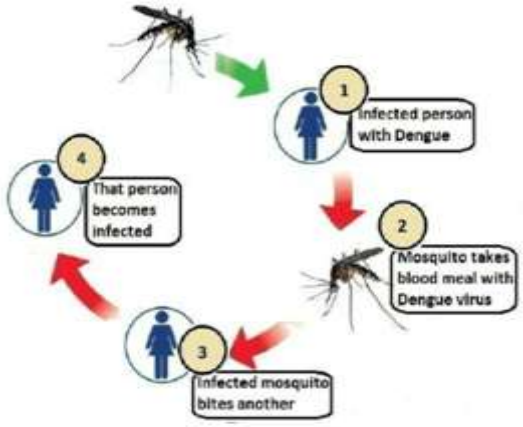

Figure 1: Example of mosquito's role as a vector in disease transmission. Mosquitoes become a vector for the Dengue Virus by feeding on an infected human host and then feeding on an uninfected human. Adapted from Sampath Aruna Pradeep and Ma, 2015.

Vector Control Methods

The growing burden of these diseases has led to research into the development of ways to treat the diseases or control the rate at which they are spread. Nothing substantial has been discovered to fully limit mosquitoes' effects on society. So far, immunizations, therapeutics, and vaccines are the main preventative and interventive methods developed to combat the illnesses caused by some mosquito-spread pathogens. Some vaccines have been proven effective in protecting people during exposure, as have some immunizations in providing effective long-term protection (Clements et al., 2010; Edelman, 2005; Kim et al., 2018). Currently, there are no cures for mosquito-borne diseases, and effective vaccines are also not widely available; thus, vector control is important for disease prevention. To control the prevalence of mosquitoes, the use of insecticide, transgenic mosquitoes, or Wolbachia-infected mosquitoes has been incorporated (Benelli et al., 2016; Shragai et al., 2017). However, their use has been deemed unsuccessful due to numerous factors, including a shortage of effective tools for mosquito population control and expanding urbanization, which enables the mosquitoes to continuously spread (Morrison et al., 2008).

Insecticide use for vector control was once widespread. Insecticides helped decrease the mosquito population, resulting in a decrease in cases related to disease caused by mosquito-borne pathogens. However, the prevalence of insecticide resistance in a number of mosquito species has led to the regulation of its use (Benelli et al., 2016). Additionally, some insecticides have been discovered to have adverse effects on the environment as well as non-target organisms (Benelli et al., 2016). Dichlorodiphenyltrichloroethane (DDT), for example, was once a widely used insecticide that helped curb the spread of mosquito-borne diseases; it was powerful and detrimental to the health of mosquitoes (Berg et al., 1992; van den Berg, 2009; Turusov et al., 2002). However, DDT's ability to persist in the atmosphere has caused damage to non-target organisms and the environment, thus leading to its regulation (Aneck-Hahn et al., 2006; Berg et al., 1992; Turusov et al., 2002). DDT has been discovered in eggs of predatory birds, in the soil, in fish, and even in the seminal fluids of humans (Aneck-Hahn et al., 2006; Berg et al., 1992; Matthiessen, 1985). The continuous threat it poses to human health and the environment makes it an unsuitable choice for regulating mosquitoes.

Wolbachia-infected mosquitoes, on the other hand, have been proven to be somewhat effective. Wolbachia is an intracellular bacterium which, when introduced in a mosquito, affects its lifespan as well as its ability to spread its pathogens (Aneck-Hahn et al., 2006; Berg et al., 1992; Matthiessen, 1985). The Wolbachia bacterium, in an infected mosquito, causes a decrease in the lifespan of the mosquito and also causes a disruption in the life cycle of the viruses and the pathogens in the mosquito, thereby diminishing its ability to spread its pathogens (Benelli et al., 2016; Hoffmann et al., 2011; McMeniman et al., 2009). Additionally, this intracellular bacterium, Wolbachia, causes cytoplasmic incompatibility, the inability of sperm and egg to form a viable offspring, when an infected male mosquito mates with an uninfected female mosquito. However, reproductive success is observed when an infected female mates with an uninfected male or an infected male (Benelli et al., 2016; Hoffmann et al., 2011; McMeniman et al., 2009). As more females become infected, it becomes an ineffective means of control.

No biologically safe and fully effective control tools have been created; hence, the development of new tools to control Ae. aegypti population is of critical public health importance, as control of its population leads to control of the pathogens it spreads (Morrison et al., 2008). Mating, on the other hand, has shown promise as it has been discovered to change female behavior and physiology (Adlakha and Pillai, 1975; Avila et al., 2011; Baldini et al., 2012; Downe, 1975). For example, in comparison to virgin females, mated

females have a decreased feeding frequency and are less likely to seek hosts for a bloodmeal (Fernandez and Klowden, 1995; Klowden and Lea, 1979). In order to control the mosquito population, an in-depth understanding of its reproductive biology is important.

Reproductive Biology of Aedes Aegypti

Mating and reproduction play a role in ensuring the survival of species, so fully understanding the mosquito reproductive biology will provide insight that can be used to create tools that serve as a means of population control. The process of mating (coupling, copulation, and insemination) occurs very rapidly in mosquitoes, with it taking about 10 seconds for the seminal fluid, containing sperm, to be passed from the male mosquitoes into the female mosquitoes (Carvalho et al., 2018; Fernandez and Klowden, 1995; Gwadz et al., 1971). This seminal fluid, when received by the female mosquito, leads to several responses that function to enhance fertility in the female mosquito(Avila et al., 2011; Baldini et al., 2012). These responses, which include egg development, digestion, and oviposition, have a higher rate in mated female mosquitoes when compared to virgin females (Judson, 1967; Leahy and Craig, 1965). Furthermore, mated females have a lower rate of blood-feeding, insemination by other males, as well as response to host cues(Downe, 1975; Gwadz et al., 1971; Shutt et al., 2010).

These changes observed in mated females are modulated by molecules produced in the male's accessory reproductive glands and transferred to the female in the seminal fluid during mating(Avila et al., 2011). While non-protein molecules are present in the seminal fluid,proteins are the major components. These seminal fluid proteins (SFPs) are believed to play a significant role in regulating female reproductive activity, as their absence is detrimental to reproductive success (Baldini et al., 2012; Gillott, 2003).

Determining how specific SFPs modulate female Ae. aegypti behavior and physiology can provide important insights in identifying novel pathways and lead to the creation of new tools that control mosquito populations. For example, SFPs are known to decrease the likelihood of female mosquitoes remating (Avila et al., 2011). Genetically modified female mosquitoes, which transfer a gene that codes for SFPs that inhibit re-mating to their offspring, could be created and released into the wild. This would lead to a sterile generation of female Aedes mosquitoes, as these female mosquitoes would be expressing the SFPs, and the male mosquitoes would be less likely to mate with them. These behavioral changes will prevent successful reproduction.

Adipokinetic Hormone

Over 400 SFPs have been identified in the Ae. aegypti mosquito (Avila et al., 2011; Degner et al., 2019; Sirot et al., 2008, 2011). These proteins belong to classes such as proteolysis regulators, venom allergens, cysteine-rich secretory proteins, lectins, and lipases inter alia. Among the hundreds of proteins present in the seminal fluid of a male Ae. aegypti mosquito, adipokinetic hormone (AKH), a multifunctional neuropeptide, stands out. In previous work done by Boes et. al. (2014), AKH, which is known to be present in organs such as the neuroendocrine gland in adultmosquitoes, was characterized in the male seminal fluid of Ae. albopictus(Boes et al., 2014; Gäde, 1990; Van der Horst, 2003). This discovery was very important as AKH has never been discovered in the seminal fluid of an insect before. The presence of AKH in the seminal fluid was later confirmed in Ae. aegypti by Dr. Laura Sirot (The College of Wooster), making it an SFP of interest. Further contributing to the interest in AKH is its classification as a neuropeptide; neuropeptides are known to be distributed and function in the central nervous system of insects (Holman et al., 1992). They play important roles in regulating physiological, developmental, and behavioral processes –such as feeding, courtship, digestion, sleep, learning, stress, oogenesis, and memory— in insects (Gäde, 2004; Schoofs et al., 2017). The overlap of the roles of this neuropeptide with some of the phenotypes of mated female mosquitoes solidifies an interest in this protein.

Additionally, AKH is known for its roles in influencing nutrition and reproduction-related physiological processes in invertebrates, making it a promising candidate in regulating the mated female phenotype (Kaufmann et al., 2009; Shragai et al., 2017). AKH is synthesized and stored in the corpora cardiaca, a neuroendocrine gland attached to the brain (Mutlu et al., 2018). When AKH release is signaled, it is secreted from the corpora cardiaca and binds to the AKH receptor, which then, depending on the need of the mosquito, initiates the release of energy from fat bodies harvested from food digestion, which regulates important activities such as flight and hibernation, or influences the nutritional balance during egg development (Boes et al., 2014; Kaufmann and Brown, 2008; Kaufmann et al., 2009). Understanding the role AKH plays in bloodmeal

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digestion in Ae. aegypti would provide insights that can lead to the creation of effective and environmentally friendly tools, which would be used to control their population.

Digestion in Female Aedes Aegypti Mosquitoes

Female mosquitoes, which usually feed on water and the nectar of plants, will feed on blood when they need nutrients for ovary development (Foster, 1995).Blood, which predominantly contains protein, is the meal of choice as it is a source of important nutrients needed for oogenesis. The mosquito ingests its meal using its proboscis, and the food is subsequently moved through the alimentary canal, which consists of the foregut with adjacent salivary glands, midgut, hindgut, and Malpighian tubules (Clements, 1992). The midgut is the main site of digestion, as it harbors proteolytic enzymes in its epithelial cells, which are predominantly involved in food breakdown and nutrient absorption (Clements, 1992). The activity of the proteolytic enzyme is increased by the presence of blood in the midgut (Briegel and Lea, 1975; Fisk and Shambaugh, 1952). Immediately after the mosquito feeds on the blood, its midgut is hugely distended, and it engages in diuresis for the next hour (Clements, 1992; Stobbart, 1977). A few hours later, ribosomes and endoplasmic reticulum (ER) formation and organization occur. This then leads into the phase of production of digestive enzymes and absorption of digestive products. High levels of trypsin are continuously expressed in the midgut, which allows digestion of protein present in the blood to begin (Brackney et al., 2010; Dias-Lopes et al., 2015; Noriega and Wells, 1999). The expression of trypsin in the midgut is dependent on the intake of blood; the presence of sugar or solutions cannot cause the expression of trypsin (Clements, 1992). As the blood is being digested, synthesis of yolk proteins, which are important for oocyte development, is being induced. Once produced, the yolk proteins are incorporated into the oocytes. The complete digestion of blood takes about 2 – 3 days (Gaio et al., 2011).

Digestion rate in mosquitoes is known to be influenced by temperature, humidity, the presence of sugar, and the presence of blood (Clements, 1992). An increase in temperature leads to an increase in the rate of digestive enzyme synthesis and the kinetics of the digestive enzyme. Humidity also increases the rate of digestion, while the presence of sugar inhibits the normal digestion of proteins present in the blood (Clements, 1992). Additionally, virgin and mated female Aedes mosquitoes have different digestion rates. This difference is believed to be a result of SFPs (Edman, 1970; Houseman and Downe, 1986). Virgin females have a slower rate of digestion when compared to mated females. When virgin females are injected with SFPs, their digestion rate is discovered to be higher than that of virgin females injected with saline solution (Edman, 1970; Houseman and Downe, 1986). This difference in digestion rate in virgin and mated females is believed to be attributed to the SFPs received from the male mosquito during mating. AKH is hypothesized to be the main SFP contributing to this difference, as neuropeptides are known to operate digestive processes (Bil et al., 2014). For example, neuropeptides such as proctolin, allatotropin, and tachykinins are believed to act on the gut muscles of insects, stimulating their activity during digestion, whereas allatostatins, myosuppressins, and myoinhibitory peptides are believed to inhibit it (Audsley and Weaver, 2009). Additionally, the decapitation of mosquitoes was discovered to stop bloodmeal digestion (Gulia-Nuss et al., 2011). AKH also belongs to a family of red pigment-concentrating hormones, which control fat, carbohydrate, and protein metabolism in insects (Gäde, 2009). Thus, AKH may be implicated in blood meal digestion.

Previous work done in Pyrrhocorisapterus L. (Heteroptera), firebugs, showed that AKH signaling increased the digestive activity in the midgut (Kodrík et al., 2012). In this study, the firebugs were injected with AKH through the metathoracic-abdominal intersegmental membrane into their thorax, and the effects on the midgut processes were observed. The peptidase activity in the midgut was discovered to be significantly increased when the firebugs were injected with AKH (Kodrík et al., 2012). The midgut of the firebug also showed an increase in the triacylglycerols level with food content and an increase in the diacylglycerols level when stimulated with AKH. This observation suggests that AKH might affect processes involving the digestion of food, food turnover, and lipid metabolism (Kodrík et al., 2012).

Additionally, a study done in Sarcophaga crassipalpis, flesh flies, showed that AKH increased proteolytic activity in the midgut to increase digestion rates (Bil et al., 2014). This study was done on sugar-fed flesh flies, liver-fed decapitated flesh flies, and liver-fed flesh flies with intact heads. It was observed that the midgut enzymatic activity of the decapitated flies was lower than the basic level of the sugar-fed flies, as

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sugar does not stimulate protease activity (Bil et al., 2014). When the flies, which were decapitated post-feeding, were injected with AKH, the proteolytic activities in their midgut were observed to increase to that of a liver-fed fly with an intact head. The proteolytic activity in the decapitated flies was also observed to increase with a rise in the amount of AKH injected into them (Bil et al., 2014). In the sugar-fed flies, an increase in the enzyme activity in the midgut was observed when they were injected with AKH. The digestive activity was observed to be higher than that of liver-fed decapitated flies (Bil et al., 2014).

Due to the effects of AKH on digestion rates observed in flesh flies and firebugs, it will be interesting to see if the seminal fluid-derived AKH will also increase the digestion rate of blood meal in mated female Ae. aegypti mosquitoes (Bil et al., 2014; Kodrík et al., 2012). As the individual proteins that induce the changes in digestion rate in mated female Aedes mosquitoes have never been identified, this work will help determine if AKH is one of such proteins and will help fill important gaps in scientific knowledge. This will additionally bring us a step closer to understanding the full mechanism and effect of SFPs in mated female Aedes mosquitoes, as they are yet to be fully elucidated. Being able to completely understand the role of SFPs in mated females will thus allow us to develop alternative methods, which could involve changing the female mosquitoes' physiology and behavior, that can be applied in population control.

II. EXPERIMENTAL PROCEDURES

Overview

The purpose of this study is to determine if seminal fluid-derived AKH passed to the female Ae. aegpytipost-mating has an effect on their bloodmeal digestion rate. To investigate this experiment, the normal pattern and rate of digestion in virgin females were established, then compared to that of both females mated to wild-type (WT) males and females mated to AKH-null males. The mosquito strains used in this experiment were the Thai strain, Liverpool (LVP), and AKH-null, which were obtained from Dr. Laura Sirot (The College of Wooster, Wooster, OH). The LVP mosquitoes are wildtype. The AKH-null males, which were generated via CRISPR method, were also obtained from Dr. Laura Sirot (The College of Wooster, Wooster, OH). The AKH-null males were used to test whether it was the AKH protein that was having an effect on the female mosquitoes' blood digestion rate.

In order to determine the effect of AKH on bloodmeal digestion rate in mated females, three specific aims were developed for this study. The first aim was focused on developing an efficient method for extracting the ingested bloodmeal and measuring digestion rate in mosquitoes, as it was paramount to the result. The second aim of the experiment was to confirm that SFPs increase the digestion rate as established in previous studies (Edman, 1970; Houseman and Downe, 1986). The third aim of this study was to determine if AKH is the SFP that influences bloodmeal digestion.

Methods and Materials

Hatching and rearing of Ae. aegypti eggs

In this study, the eggs, contained in egg-laying paper, were hatched in deionized (DI) water. This was done by putting the mosquito eggs, contained in egg-laying paper, in a 500mL mason jar in low dissolved oxygen water for 4hrs. The egg-laying paper with the eggs was then transferred to a plastic container, comprising 1 L distilled water and ground up Cichlid Gold Specialists' Fish pellet, and maintained at 27 °C. Two days after hatching the eggs, the developed larvae were transferred to a new plastic container with 1 L DI water and ground up Cichlid Gold Specialists' Fish pellet, and incubated at 27 °C. Each container consisted of ~200 larvae with four ground-up fish pellets. The pupae, which start developing on day 6 post-hatching, were individually transferred into small test tubes containing water from the tub and subsequently stored at 27 °C. On day 8 post-hatching, the emerged adults were released into a same-sex cage made of a 3.5 L plastic bucket with a mesh covering the top of the bucket. All cages contained 100 mosquitoes each, a wick placed in sucrose water for feeding (10% sucrose water for Thai mosquitoes, 10% sucrose water for LVP mosquitoes and 20% sucrose water for AKH-null mosquitoes) and were kept at 27 °C with a relative humidity of >50% and a 12:12 light:dark cycle at all life stages. In some cases, a total of 100 pupae were kept in a 20mL plastic container with DI water. This plastic container was placed in the plastic bucket cage with sucrose water as described above. The adults that emerged from these pupae were thus in a mixed-sex cage.

Mating protocol

The mosquitoes were mated in the morning, starting from 8am. The humidifier was turned on prior to mating to ensure the room reached a humidity greater than 50%. Using a mouth aspirator, adult female mosquitoes (4-11

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

days old) were individually placed in a cage containing 20 adult male mosquitoes. The female mosquitoes were carefully watched to ensure that they mated with a male for at least six seconds after their genitalia connected. The females that successfully mated were transferred to a new cage, while the unsuccessful females were not used. The mating groups included virgin LVP female mosquitoes (treatment 1), LVP females mated to LVP males (treatment 2), and LVP females mated to AKH-null males (treatment 3). The matings were carried out in the months of November and December.

Blood feeding and freezing protocol

The mosquitoes were bloodfed the day after mating. The sucrose water was removed from their cages about 10-12hrs prior to bloodfeeding. To simulate the appropriate environment for blood feeding for mosquitoes, the humidifier in the mosquito room was switched on prior to feeding to allow the humidity of the room to reach above 50%. The bovine blood for feeding was prepared by mixing about 10mg of ATP with about 3mL of blood in a glass bowl. This was then transferred to a blood feeding cylinder with a membrane covering. The cylinder was subsequently connected to the Hemotek blood feeding apparatus to allow the blood to warm up to 37 °C. The cylinder was put on top of the cage with the membrane facing the mosquitoes for one hour to allow the mosquitoes ample time to feed. The mosquitoes were allowed to bloodfeed from 8pm to 9pm. The blood-fed mosquitoes were aspirated in groups of four into test tubes and kept in the 27 °C incubator. The bloodfed mosquitoes were transferred into the -20 °C freezer at 0, 6, 12, 16, and 20hrs post-feeding. At the respective times post feeding, the mosquitoes were transferred to a -20 °C freezer. The mosquitoes were bloodfed during the months of August, September, October, November, and December.

Bloodmeal Extraction

All surfaces were wiped down with 70% ethanol. The frozen mosquitoes were obtained from the -20 °C freezer and kept in a tub of ice to keep the proteins from degrading. A petri dish containing ice was kept under the light of the microscope with a slide on it. The slide had four 6μL drops of dissecting cocktail (1 Protease inhibitor tablet (cOmplete Mini, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany) dissolved in 1X PBS) on it. The mosquito was removed from the tube and dissected by detaching the head and thorax of the mosquito from the abdomen using forceps. The wings and hind and mid legs were also removed to prevent sample contamination. The blood in the abdomen of the mosquito was gently squeezed out of the anterior of the midgut, into a drop of dissecting cocktail on the slide using forceps. The bloodmeal in the dissecting cocktail on the slide was transferred to a 600μL Eppendorf tube using a micropipette. The bloodmeal was then homogenized using a pestle, and the tubes were then topped off to 20 μL using sample buffer (2x Laemmli sample buffer mixed with β-mercaptoethanol). The samples were boiled for 4 minutes and then transferred to the -20 °C freezer.

SDS-PAGE and Coomassie Staining

The samples were boiled for 4mins. They were then run through an SDS-PAGE (4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels) at 200V for 30mins. A control, comprised of a 1:3 ratio of bovine blood, was also run through the gel to be able to determine the bands that correspond to the bovine blood protein present in the bloodmeal extracted from the mosquitoes' midgut. The ladder used was BIO-RAD Precision Plus Protein™ Standard and the running buffer used was a 1X Tris Glycine SDS buffer. After running the gel for 30 mins, it was gently removed from the cassette and washed three times in 100 mL of DI water for 5 minutes during each wash. The gel was then stained for 1hr with 20 mL of Coomassie Blue. After staining, the gel was washed in 100 mL of DI water two times for a minimum of 1hr and a maximum of 1hr 10 mins during each wash. The gel was then viewed under the Bio-Rad imager, under the Coomassie setting.

Data Analysis

The Bio-Rad ChemiDoc MP was used to visualize the bands present in the Coomassie Stained SDS-PAGE gel. The intensity of the bands and the whole lane was determined using the ImageJ software. This was done by fitting the band/lane of interest into a rectangular box and then analyzing the integrated density of the area covered by the box. R was used to create graphs of the average intensities of the various bands as well as the whole lane. R was also used to carry out a paired t-test of the data. This was done to determine if there was a significant change in intensity over time. The formula: [(Intensity at 20hrs-Intensity at 12hrs)/Intensity at 12hrs] ×100%— was used to calculate the percent change over time (Table 2). No t-test was done on the percent change as a small

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sample size was worked with; hence, the data was not normal.

III. RESULTS

To accomplish the first aim of this experiment, various methods of extracting bloodmeal were first analyzed. Methods such as grinding up the entire mosquito to extract the bloodmeal and rupturing the abdomen of the mosquito were determined to be inefficient for this experiment, as they can lead to contamination of the sample and therefore skew the results. Thus, the method of extracting bloodmeal used in this experiment included removing the head, legs, and thorax from the abdomen of the mosquito, and then gently squeezing the bloodmeal out through the anterior of the mosquito's midgut, as described in the methods.

After determining the appropriate method of dissection, the right amount of extracted bloodmeal to load in the gel was determined. To decide the appropriate amount of bloodmeal to run through the gel, a dilution series, using sample buffer mixed with β-mercaptoethanol, wasdone on the samples obtained (Figures 2, 3 & 4). Full mosquito bloodmeals at different timeintervals proved to be problematic when run on a gel (Figures 2 and 3). The signals of their bands were so bright that clear and distinct bands in their lanes could not be made out. Additionally, the proteins in the lanes corresponding to full mosquito bloodmeal were so concentrated that they appeared wide on the gel, affecting the ability of the samples in the lanes next to them to run properly. A similar observation was made in the lanes corresponding to ½ bloodmeal. The same conclusion was not drawn for ¼ of a bloodmeal. When looking at Figure 2, which had ¼ of a bloodmeal, it was determined that the bands in lanes 4

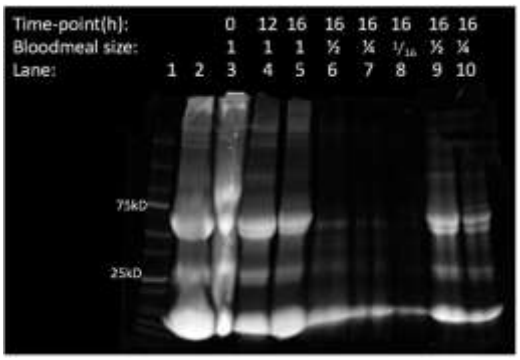

Figure 2:Coomassie-stained SDS-PAGE of bovine blood and bloodmeals of various amounts obtained from Thai mosquitoes (16-18 days old) at various time intervals (n=9).The gel was run for 45mins at 120V. Lane 1- BIO-RAD Precision Plus ProteinTM and a 4-20% Mini-PROTEAN TGX Stain-FreeTM Protein Gels. Mosquitoes used were mated as they were obtained from a mixed-sex cage.

and 8 were clear. They looked similar and consistent with each other as they all had the same number of bands. Thesignals of the bands were also strong and not overwhelming. They did not influence or affect the samples running in the lanes next to them. Furthermore, when observing the lanes corresponding to $^1/_8$and$^1/_{16}$ of a bloodmeal, it was determined that the bands presen in the lanes were too faint to clearly visualize the bands present in the bloodmeal. Thus, ¼ of the mosquito bloodmeal was determined to be an appropriate amount to be run on the gel. Additionally, bovine blood, which was also run through an SDS-PAGE, helped in visualizingthe bands expected to be present in the bloodmeal extracted from the mosquitoes' midguts (Figure 2). Two bands appeared at around 75kD, two bands appeared at about 25kD and one band appeared at 10kD. These bands appeared consistently throughout the gel in lanes corresponding to the various amounts of bloodmeal sample obtained from the various treatment groups at different time intervals. This observation suggests that sample contamination did not occur as the bands seen when the extracted bloodmeal is run through an SDS-PAGE are from the ingested bloodmeal, and not the mosquito.

The appropriate timepoints post-feeding in which there is an obvious difference in the degradation of the protein in bloodmeal were then determined in order for the digestion rate to be measured. This was done by

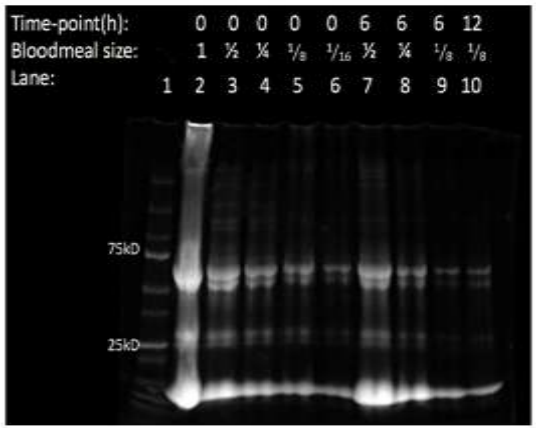

Figure 3: Coomassie-stained SDS-PAGE of bloodmeals of various amounts of obtained from

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Thai mosquitoes (19-23 days old) at various time intervals (n=9). The gel was run for 45mins at 120V. Lane 1- BIO-RAD Precision Plus ProteinTM and a 4-20% Mini-PROTEAN TGX Stain-FreeTM Protein Gels. Mosquitoes used were mated as they were obtained from a mixed-sex cage.

freezing the female mosquitoes at various timepoints post-feeding and running an SDS-PAGE of the bloodmeal extracted from their midgut (Figures 2, 3 & 4). When 6 and 12hrs post-feeding were compared to each other (Figures 2 & 3), the bands present were determined to be too similar to look at the changes in bloodmeal proteins over time. This same conclusion was drawn when 12 and 16hrs were compared to each other (Figure 5). However, when 12 and 20hrs post-feeding were compared to each other (Figure 4), the bands above 25kD appeared to go from two bands in 12hrs post-feeding (lane 5; Figure 4) to one band 20hrs post feeding (lane 4; Figure 4). The brightness of the bands at about 75kD also appeared to decrease from 12hrs to 20hrs, while the brightness of the 10kD bands appeared to increase from 12 to 20hrs. Thus, 12 and 20hrs post-feeding were determined to be the appropriate timepoints to compare the change in bloodmeal digestion across treatment groups.

Various methods were analyzed in order to determine an effective way of measuring bloodmeal digestion rate. The intensities of the whole lane, in which the samples were run, were first examined. The expectation was that there would be a decrease in intensity over time as digestion would have progressed and more proteins would have been degraded. This however was not the case as there was no significant difference between the intensity of the whole lane at 12, and 20hrs ($t = -0.001$, $p = 1.00$, $df = 11$; Figure 6; Table 1). The percent change of the intensity over time, which was calculated using the formula: [(Intensity at 20hrs-Intensity at 12hrs)/Intensity at 12hrs] ×100%, of the whole lane across various treatment groups was analyzed, and no trend was observed (Table 2). The percent change of the virgin group ranged from -36% to 34.2%, the percent change in the group with LVP females mated to LVP males ranged from -57.5% to 138%, while the percent change of the group of LVP females mated toAKH-null males ranged from -41.5% to 51.7% (Table 2). There was no consistency in the percent change of the average intensity of the whole lane. The intensities of the 25, and 75kD bands were determined not to be good indicators for the digestion rate (discussed in the appendix).

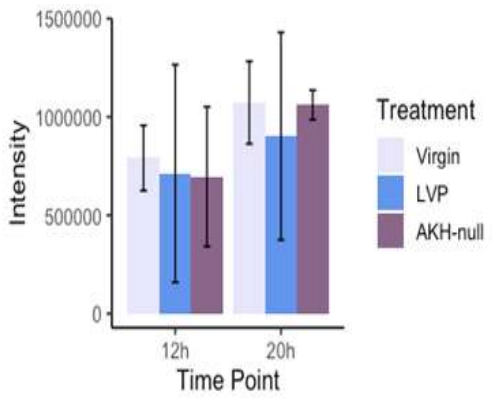

Figure 4: Bar graph of the intensity of the 10kD bands of the various treatment groups at different time intervals (obtained from SDS-PAGE).The lavender coloured bar corresponds to the LVP virgin mosquitoes, the blue coloured bar corresponds to the LVP females mated to LVP males while the plum coloured bar corresponds to LVP females mated to AKH-null males. The error bars represent the standard deviation of the intensities in each treatment group. 12h and 20h represent the mosquitoes frozen 12hrs and 20hrs after feeding.

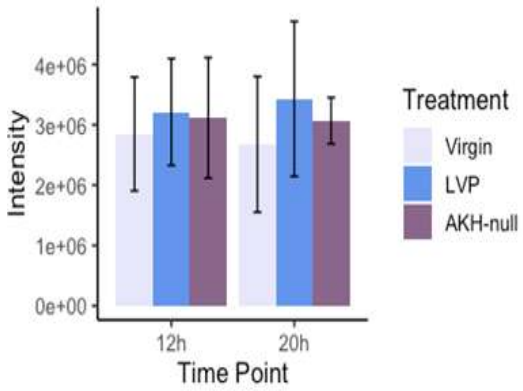

Figure 5: Bar graph of the average overall intensity of the lanes of the various treatment groups at different time intervals (obtained from SDS-PAGE). The lavender-coloured bar corresponds to the LVP virgin mosquitoes, the blue coloured bar corresponds to the LVP females mated to LVP males, while the plum coloured bar corresponds to LVP females mated to AKH-null males. The error bars represent the standard deviation of the intensities in each treatment group. 12h and 20h represent the mosquitoes frozen 12hrs and 20hrs after feeding.

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

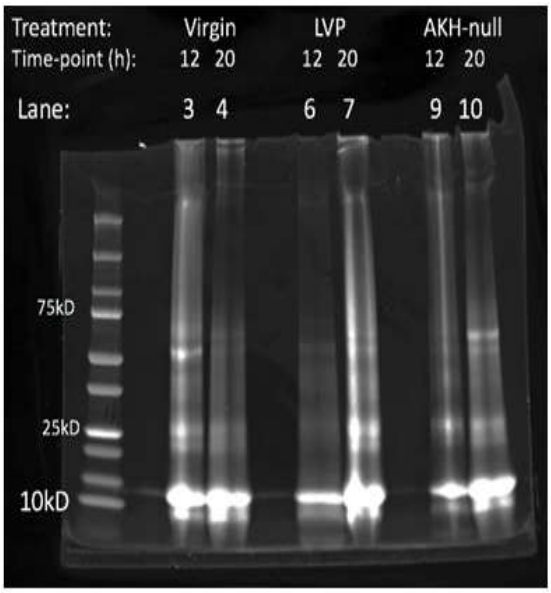

Figure 6: Coomassie-stained SDS-PAGE of bloodmeals from the various mosquito treatment groups (4-11days old) at different time intervals (n=6).The gel was run for 30mins at 200V. The various treatment groups and time points are indicated at the top of each lane. One quarter of the extracted bloodmeal was run in the lanes. Virgin represents the LVP virgin mosquitoes, LVP represents LVP females mated to LVP males while AKH-null represents LVP females mated to AKH-null males. The ladder used was BIO-RAD Precision Plus ProteinTM Standard. One quarter of a bloodmeal was run in the lanes.

The intensities of the 10kD bands were also analyzed. The intensity of the 10kD band was observed to increase over time in all the treatment groups except for the females mated to AKH-null males in Figure 9 (Table 1). Additionally, there was a significant difference between the average intensity of the 10kD bands at 12 and 20hrs ($t = -3.24$, p-value = 0.008, df=11; Figure 5). The average intensity of the 10kD band was, however, not used to compare the digestive rate among the various treatment groups because the intensity is dependent on the bloodmeal size of the individual mosquitoes, since the samples loaded in the SDS-PAGE were not quantified. The percent change over time of the intensity of the 10kD band across various treatment groups was thus analyzed. The average percent change across all treatment groups ranged from 41.4 – 89.4% (Table 2).

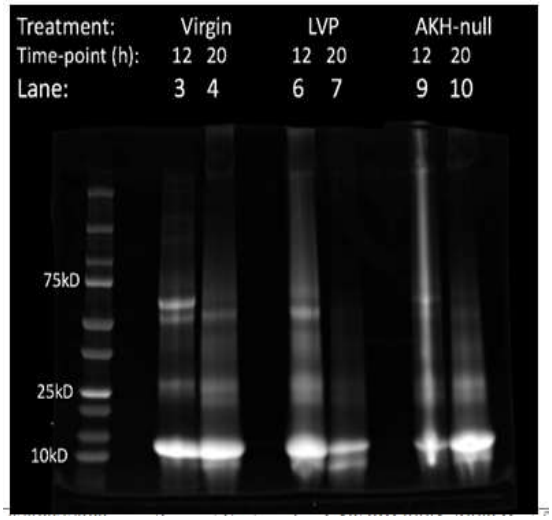

Figure 7: Coomassie-stained SDS-PAGE of bloodmeals from the various mosquito treatment groups (4-11days old) at different time intervals (n=6). The gel was run for 30mins at 200V. The various treatment groups and time points are indicated at the top of each lane. One quarter of the extracted bloodmeal was run in the lanes. Virgin represents the LVP virgin mosquitoes, LVP represents LVP females mated to LVP males while AKH-null represents LVP females mated to AKH-null males. The ladder used was BIO-RAD Precision Plus ProteinTM Standard. One quarter of a bloodmeal was run in the lanes.

The second aim, which served to confirm that SFPs increase the digestion rate as established in previous studies, was investigated by analyzing the data obtained from virgin females and females mated to WT males. It was hypothesized that there would be a higher average percent change in the 10kD over time in samples obtained from the females mated to WT males, when compared to the samples obtained from virgin females. The average percent change of the 10kD band present in the sample obtained from the virgin females was 41.4%, while that obtained from the females mated to WT males was 56.3% (Table 2).

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

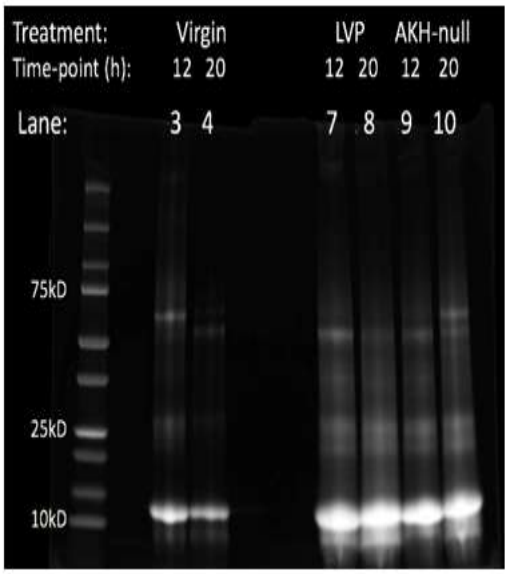

Figure 8:Coomassie-stained SDS-PAGE of bloodmeals from the various mosquito treatment groups (4-11days old) at different time intervals (n=6). The gel was run for 30mins at 200V. The various treatment groups and time points are indicated at the top of each lane. One quarter of the extracted bloodmeal was run in the lanes. Virgin represents the LVP virgin mosquitoes, LVP represents LVP females mated to LVP males while AKH-null represents LVP females mated to AKH-null males. The ladder used was BIO-RAD Precision Plus ProteinTM Standard. One quarter of a bloodmeal was run in the lanes.

To examine aim three, which functioned to determine if AKH was the protein increasing bloodmeal digestion in mated females, the data obtained from the females mated to AKH-null males were compared to that obtained from the virgin females and that obtained from the females mated to WT males (Figures 7, 8, 9 & 10). It was hypothesized that the percent change of the 10kD band in females mated to AKH-null males would be lower than that of the females mated to WT males and either be the same as or higher than that of the virgin females. When comparing the average intensity of the virgin females and the females mated to AKH-null males, it was determined that the average percent change of the AKH-null females, 89.4%, was higher than that of the virgin females, 41.4%. Furthermore, when the average percent change of the femalesmated to AKH-null males was compared to the females mated to WT males, it was discovered that the average percent change of the latter, 56.3%, was higher than that of the former, 89.

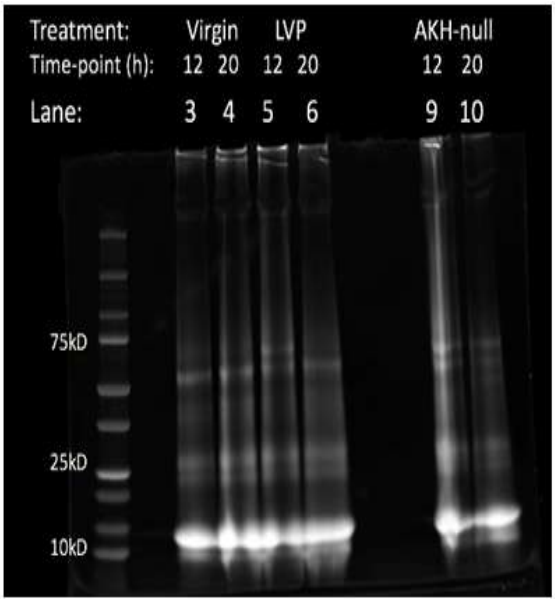

Figure 9:Coomassie-stained SDS-PAGE of bloodmeals from the various mosquito treatment groups (4-11days old) at different time intervals (n=6).The gel was run for 30mins at 200V. The various treatment groups and time points are indicated at the top of each lane. One quarter of the extracted bloodmeal was run in the lanes. Virgin represents the LVP virgin mosquitoes, LVP represents LVP females mated to LVP males while AKH-null represents LVP females mated to AKH-null males. The ladder used was BIO-RAD Precision Plus ProteinTM Standard. One quarter of a bloodmeal was run in the lanes.

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

Table 1. Summary of the Intensities of the 10kD bands.

TREATMENT	Virgin	Virgin	LVP	LVP	AKH-null	AKH-null
Time Point	12hrs	20hrs	12hrs	20hrs	12hrs	20hrs
Figure 7						
	Lane 3	***Lane 4***	***Lane 6***	***Lane 7***	***Lane 9***	***Lane 10***
Whole Lane	2794234	3214947	3944726	1675880	3177551	2952898
Last Band	956540	1089877	74549	136498	365102	1142262
Figure 8						
	Lane 3	***Lane 4***	***Lane 6***	***Lane 7***	***Lane 9***	***Lane 10***
Whole Lane	4053979	2596108	1934541	4612029	2350472	3550797
Last Band	742111	773536	432868	1000365	466169	1088817
Figure 9						
	Lane 3	***Lane 4***	***Lane 6***	***Lane 7***	***Lane 9***	***Lane 10***
Whole Lane	1748370	1145545	3355540	3325473	2425010	3137359
Last Band	581446	1246628	1246628	1324969	1147470	1047517
Figure 10						
	Lane 3	***Lane 4***	***Lane 6***	***Lane 7***	***Lane 9***	***Lane 10***
Whole Lane	2795773	3751560	3615063	4110839	4504460	2633422
Last Band	884313	1180802	1095483	1147884	807654	964067

Note: Virgin refers to the LVP virgin females, LVP refers to LVP females mated to LVP males and AKH-null refers to LVP females mated to AKH-null males.

Table 2: Change in Intensity of the whole lane and the 10kD band under various over time.

TREATMENT	**Figure 7**	**Figure 8**	**Figure 9**	**Figure 10**	
Whole Lane					**Average**
Virgin	15.1%	-36.0%	-34.5%	34.2%	-5.30%
LVP	-57.5%	138%	-0.90%	12.7%	23.1%
AKH-null	-7.10%	51.7%	29.4%	-41.5%	8.13%
10kD Band					**Average**
Virgin	13.9%	4.23%	114%	33.5%	41.4%
LVP	83.1%	131%	6.28%	4.78%	56.3%
AKH-null	212%	134%	-8.71%	19.4%	89.4%

Note: Virgin refers to the LVP virgin females, LVP refers to females mated to LVP males, and AKH-null refers to LVP females mated to AKH-null males

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IV. DISCUSSION

Overview

AKH is a neuropeptide that is known to play a role in the metabolism of energy stores (Gäde, 2004; Holman et al., 1992; Van der Horst, 2003). The presence of AKH in the seminal fluid of Aedes albopictus, discovered by Boes et al. (2014),its confirmation in Aedes aegypti by Dr. Laura Sirot (The College of Wooster, Wooster, OH),as well as its role in the metabolism of energy, led to the notion that it may be implicated in the digestion of bloodmeal (Boes et al., 2014; Kaufmann and Brown, 2008). Additionally, AKH has been determined to play a role in female insects' bloodmeal digestion. It is known to increase the digestive activity in the midgut of firebugs and flesh flies (Bil et al., 2014; Kodrík et al., 2012). However, its role in mosquitoes' bloodmeal digestion is unknown. This study was done to determine the effect AKH has on bloodmeal digestion. Female mosquitoes are less likely to feed if their meal has not been fully digested (Clements, 1992). If AKH increased the rate of bloodmeal digestion, as observed in other species, its effects in female mosquitoes' bloodmeal digestion would be blocked, thus decreasing the rate at which bloodmeal is digested, leading to a decreased feeding frequency.

Methodology for Bloodmeal Extraction and Data Analysis

To be able to investigate the role of AKH in bloodmeal digestion, establishing an effective methodology in bloodmeal extraction and protein analysis was the main focus of the first aim. In a study done by Irby and Apperson (1989), analysis of human and rodent blood by Aedes aegypti was performed.Their methodology involved grinding up the full mosquitoes in order to extract their bloodmeal. This method of bloodmeal extraction was viewed as not being fully effective, as other proteins in the mosquitoes could have influenced the digestion of the bloodmeal and contaminated the sample. Additionally, when looking at the figures in their study, the immunoblot shows an inconsistency in the protein bands present at some timepoints post-feeding. Therefore, an alternative method of extraction was used in this study. In a study done by Bil et al., (2014),the method used for extracting bloodmeal involved dissecting out the midgut from the flesh fly and homogenizing the bloodmeal in Ringer solution. This method was partly incorporated into mosquito bloodmeal extraction. In this study the thorax, legs and wings were detached from the mosquito and the bloodmeal was gently squeezed from the anterior of the mosquito's abdomen, into a solution of dissecting cocktail (1 Protease inhibitor tablet dissolved in 1X PBS) and homogenized in sample buffer (2x Laemmli sample buffer mixed with β-mercaptoethanol) to inhibit further protein degradation. This method was used as it minimized contamination and ensured consistency in the proteins present in each treatment group at each time point, as degradation of the proteins present in the bloodmeal would be decreased.

In addition to finding an appropriate method for bloodmeal extraction, determining the amount of bloodmeal samples to run in the SDS-PAGE, the appropriate time point to freeze the mosquito post-feeding, as well as the way to measure the digestion rate, were part of the first aim. One quarter of a bloodmeal sample was determined to be optimal, as the Coomassie-stained SDS-PAGE of this amount showed good-sized bands that were not too faint and did not interfere with the running of the samples on the neighboring lanes. The time points were determined such that a change in the pattern of the bloodmeal sample is observed. This change was observed when comparing the Coomassie-stained SDS-PAGE of 12, and 20hrs post-feeding (Figure 4). The brightness of the bands at about 75kD appeared to decrease from 12hrs to 20hrs. Also, at about 25kD, the bands appeared to go from two bands at 12hrs (lane 5; Figure 4) to one band at 20hrs (lane 4; Figure 4). The overall intensities of the 12 and 20hrs lanes were analyzed and no significant difference was observed, so it was not used in data analysis. The 25 and 75kD bands were analyzed next but were determined not to be good indicators of digestion (discussed in appendix). The 10kD bands were thus analyzed next because they contain the smallest-sized proteins. It was hypothesized that the intensity of the bands would increase over time as digestion would have progressed more over time, and thus the intensity of the 10kD bands at 20hrs should have been higher than that of the 10kD band at 12hrs. This was observed as the intensity of the 10kD band was higher across all treatment groups except for the females mated to AKH-null males in Figure 9 (Table 1). Additionally, the difference in intensity of the 10kD band at 12 and 20hrs was significant. Thus, 12 and 20hrs were determined to be good timepoints to use to measure digestion rate, and the intensity of the 10kD band was used to quantify the rate. The 10kD band served as a good indicator of digestion rate as it has a consistent change over time. However, because the sample loaded was not

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

quantified, the intensity of the protein is dependent on the bloodmeal size of the mosquitoes in the various treatment groups. Thus, the percent change, calculated using the formula: [(Intensity at 20hrs-Intensity at 12hrs)/Intensity at 12hrs] ×100%, was used because it factored in the difference in the bloodmeal size and measured the relative change in the intensity over time.

Implications of SFPs in Bloodmeal Digestion

The second aim of this study served to confirm that mated mosquitoes have a faster digestion rate, as established in previous studies (Edman, 1970). Previous studies showed that SFPs increase the digestion rate of female mosquitoes (Downe, 1975; Edman, 1970). Virgin female mosquitoes had the lowest digestion rate when compared to mated females and virgin females injected with SFPs. Additionally, the was no significant difference between the digestion rate of mated females and virgin females injected with SFPs (Edman, 1970). It was therefore hypothesized that the average percent change of the virgin females would be lower than that of the females mated to WT males. When looking at the average percentage change, virgin mosquitoes were discovered to have a lower percentage change (41.4%) when compared to that of mated females (56.3%). This means that mated females do have a higher digestion rate, as the higher presence of smaller protein over time suggests that protein degradation is occurring at a faster rate.

AKH may not be Implicated in Bloodmeal Digestion

In order to determine if AKH plays a role in bloodmeal digestion, the digestion rate of females mated to AKH-null males was compared to females mated to WT males and virgin female mosquitoes. The fact that AKH is implicated in the metabolism of energy, as well as the fact that AKH was discovered to increase the digestion rate of flesh flies and firebugs led to the belief that it serves to increase digestion rate in mated mosquitoes as it has never been discovered in the seminal fluid of any insect, besides Aedes albopictus and Aedes aegypti (Boes et al., 2014; Dr. Laura Sirot, The College of Wooster, Wooster, OH). It was hypothesized that the females mated to WT males would have the highest percent change, while the virgin females would either have the lowest average percent change or their average percent change would be the same as that of the females mated to AKH-null males. This was, however, not the case. The data showed that the females mated to AKH-null males (89.4%) had a higher percent change when compared to virgin females (41.4%) and females mated to WT males (56.3%). This observation was very surprising, as the data showed the exact opposite of what was expected. It suggests that AKH might not be the SFP influencing digestion, and that taking away AKH from the females increases the digestion rate. This observation was interesting and should be explored further.

The difference in the observed data and the data from previous studies is not too surprising, as the methodology was different. In the previous studies that showed that AKH increased bloodmeal digestion rate in flesh flies and firebugs, the AKH used was chemically synthesized and not derived from the seminal fluid (Bil et al., 2014; Kodrík et al., 2012). Additionally, these studies injected AKH into the female insects in their experiments, which means that they were increasing the AKH to levels above normal, in comparison to this study, where the gene responsible for secreting AKH in the malemosquitoes (AKH-null) was taken away; thus, the mosquitoes were unable to secrete AKH.

Experimental Variations and Unknowns

The analysis of the data is not concrete, as there are variations within each treatment group; the percent change is not consistent within each treatment group (Table 2). There are several factors that could have caused variation during this experiment, thus influencing the data. The time at which the mosquitoes last fed on sucrose water before bloodfeeding is inconsistent. Some mosquitoes could have fed on sugar water three hours before the sugar water was removed from their cages, while some could have fed eight hours before the sugar water was removed from their cages. Thus, a mosquito that fed on sugar water more recently may not have had its sugar water meal fully digested and would therefore feed less on the blood. This would, in turn, lead to a lower amount of bloodmeal being extracted from the midguts of mosquitoes, or it could lead to the bloodmeal being further along in the process of digestion as it has less blood, when compared to mosquitoes that took a full bloodmeal. Consequently, the amount of bloodmeal run on the SDS-PAGE is not consistent, leading to varying intensities within each group. To correct this inconsistency and improve the methodology, the extracted bloodmeal should be quantified via Bradford assay, so the amount of protein run in each lane is the same. Additionally, there are many unknowns about bloodmeal digestion in female

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

mosquitoes that prevent the interpretation of the data from being conclusive. For example, the exact point at which SFPs start acting on digestion is unclear. The exact number of SFPs acting on bloodmeal digestion has also not been fully elucidated. The time at which SFPs begin to influence bloodmeal digestion is also unknown. Since not much work has been done in quantifying the bloodmeal digestion rate in female mosquitoes, a graph was created to hypothetically represent how the intensity of the 10kD band is expected to change over time. As digestion progresses over time, the intensity of the 10kD band increases until it reaches its peak (point B; Figure 11), after which it starts decreasing. This is because proteins are continuously being degraded as digestion progresses. Therefore, decrease in the intensity of the 10kD band after point B occurs because there are degraded proteins absent from the 10kD band because they are either smaller than 10kD or have degraded completely. It would therefore be interesting to repeat this study while freezing mosquitoes from the three treatment groups every half-hour. This would make it easy to pinpoint the time at which there is a change in digestion rate due to the influence of SFPs.

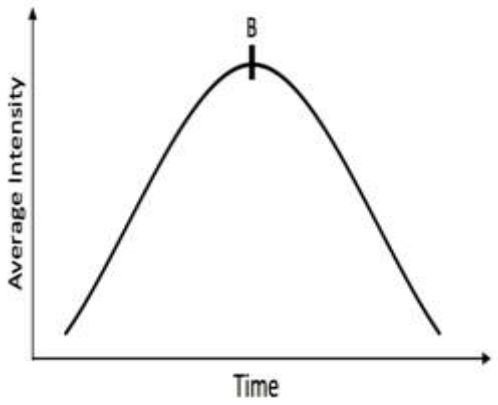

Figure 10:Expected graph of the average intensity of the 10kD band as digestion progresses further. The curve represents the average intensity of the 10kD band over time.

V. SUMMARY AND CONCLUSION

An effective method of extracting bloodmeal samples from mosquitoes involves detaching their abdomen from the rest of their body, after which the bloodmeal is gently squeezed out of the anterior of the abdomen. Using the ¼ of the extracted bloodmeal shows good sized bands when run through an SDS-PAGE; however, quantifying the protein would ensure consistency in the amount of protein run throughout the sample. When the protein is run, five bands consistently show up, two at 75kD, two at 25kD and one at 10kD. Out of all these five bands, the 10kD band was discovered to be a good indicator of bloodmeal digestion as it consistently changes from 12 to 20hrs and the intensity at the two time points are significantly different. SFPs increase the digestion rate while the absence of AKH appears to increase the digestion rate.

Since it has been re-confirmed that SFPs do influence the bloodmeal digestion rate, future studies would involve investigating ways in which SFPs increase the digestion rate. One way would be by going through the previous studies to investigate other SFPs that may be implicated in bloodmeal digestion (Boes et al., 2014; Degner et al., 2019). Transgenic mosquito lines, null for these proteins, would be created. The digestion rate of virgin females and females mated to WT males would be compared to that of the females mated to the males of these null protein lines. For example, the heat shock protein, an SFP, is a good candidate as it plays a role in protein degradation (Boes et al., 2014; Degner et al., 2019). Another direction for future work would be investigating the mechanism by which SFPs increase the digestion rate. The proteolytic activity of trypsin, which is the major protein induced by bloodfeeding, would be measured to see if it is increased in mated females when compared to virgin females (Briegel and Lea, 1975; Dias-Lopes et al., 2015; Fisk and Shambaugh, 1952; Noriega et al., 2002).

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APPENDIX

Analysis of 25kD and 75kD Bands

When looking at the gels, there appear to be five bands in each lane that correspond to the LVP virgin mosquitoes (Figures 5, 6 7 & 8). The first four bands (which range from 75 – 25kD) in each lane are somewhat weak, while the 10kD band, at the bottom of the gel, is very strong. The intensities of the whole lanes that correspond to the LVP virgin do not show a clear pattern. Furthermore, when looking at the lanes that correspond to the females mated to WT, the number of bands in each lane ranges from 3 – 6 (Figures 7, 8, 9 & 10). The 10kD band also has the highest

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Volume 7, Issue 08 Aug. 2025, pp: 31-50 www.ijaem.net ISSN: 2395-5252

intensity in comparison to the other bands in the lane. The same conclusion was drawn from observing the lanes that correspond to the females mated to AKH-null males. The number of bands in the lanes range from 5 – 6, with the 10kD band having the highest intensity.

The intensities of the other bands present in the lanes, at 12 and 20hrs post-feeding, were analyzed in addition to that of the 10kD bands and the whole lane (Figure 7, 8, 9 & 10). Most lanes contained two bands present at 75kD, named band 1 and 2, and two bands, named band 3 and 4, present at 25kD. The intensity of band 1 showed no clear pattern. In some treatment groups, band 1's intensity decreased over time while in some it increased over time (Table 3). No treatment group had a consistent increase or decrease in intensity over time. The average intensity of band 1 across Figures 7 -10 showed a decrease in intensity over time (Figure 12). At 12hrs, treatment 1 had the highest intensity while treatment 3 had the lowest average intensity. Treatment 3 had the highest intensity at 20hrs while treatment 1 had the lowest at the same time point. The differences in the intensity of band 1 at both time points were not significant (t = 1.4664, df = 11, p-value = 0.1705).

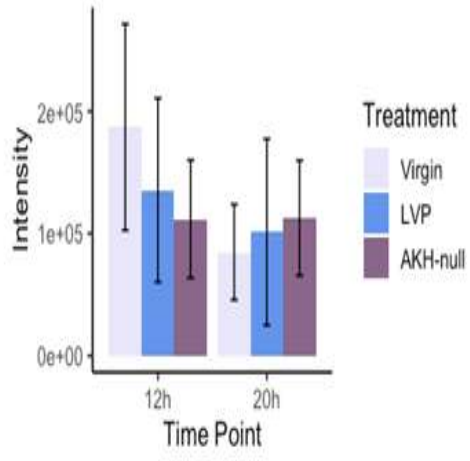

Figure 11: : Bar graph of the intensities of band 1 of the various treatment groups at different time intervals (obtained from SDS-PAGE). The lavender coloured bar corresponds to the LVP virgin mosquitoes, the blue coloured bar corresponds to the LVP females mated to LVP males while the plum coloured bar corresponds to LVP females mated to AKH-null males. The error bars represent the standard deviation of the intensities in each treatment group. 12h and 20h represent the mosquitoes frozen 12hrs and 20hrs after feeding.

The difference in the intensities of band 2 at both time points were also discovered to be insignificant (t = 1.7652, df = 11, p-value = 0.1052) (Figure 12). The intensities of band 2 over time showed no clear pattern as there was no consistent increase or decrease in its intensity over time across the various treatment groups (Table 3). Treatment 2 had the highest average intensity of band 2 at 12 hours while treatment 2 had the lowest. At 20hrs, the average intensity of band 2 in the three treatment groups was about the same with treatment 3 being the lowest. The ratio of the intensities of band 1 and 2 were investigated in order to see if a pattern could be established. There was no constant increase or decrease in their ratios over time across all treatment groups.

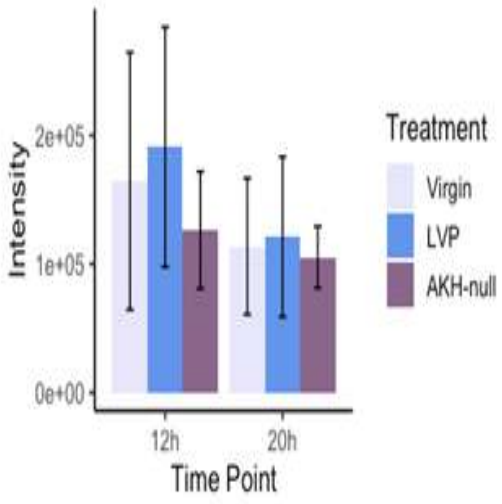

Figure 12: Bar graph of the intensities of band 2 of the various treatment groups at different time intervals (obtained from SDS-PAGE). The lavender coloured bar corresponds to the LVP virgin mosquitoes, the blue coloured bar corresponds to the LVP females mated to LVP males while the plum coloured bar corresponds to LVP females mated to AKH-null males. The error bars represent the standard deviation of the intensities in each treatment group. 12h and 20h represent the mosquitoes frozen 12hrs and 20hrs after feeding.

International Journal of Advances in Engineering and Management (IJAEM)
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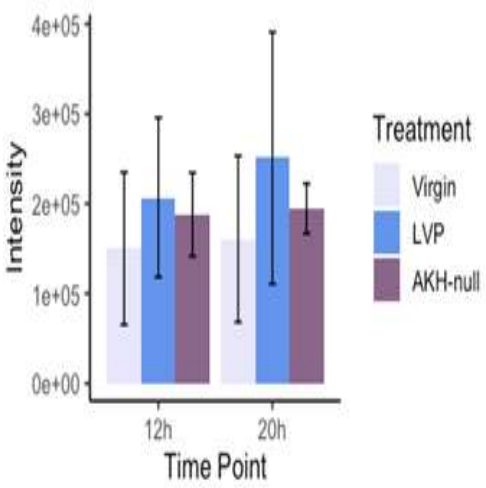

Figure 13: Bar graph of the intensities of band 3 of the various treatment groups at different time intervals (obtained from SDS-PAGE). The lavender coloured bar corresponds to the LVP virgin mosquitoes, the blue coloured bar corresponds to the LVP females mated to LVP males while the plum coloured bar corresponds to LVP females mated to AKH-null males. The error bars represent the standard deviation of the intensities in each treatment group. 12h and 20h represent the mosquitoes frozen 12hrs and 20hrs after feeding.

Band 3 and 4 also did not show promising data. The intensity of band 3 and 4 across the various treatment groups did not consistently increase or decrease over time (Table 3). At 12hrs and 20hrs, the average intensity of band 3 was highest in treatment 2 and lowest in treatment 1 (Figure 13). However, for band 4, the average highest intensity was highest in treatment 1 and lowest in treatment 3 at 12hrs (Figure 14). At 20hrs, treatment 1 had the lowest average intensity of band 1 while treatments 2 and 3 had the same average intensity. The difference in the intensities of band 3 ($t = -0.53997$, $df = 11$, p-value = 0.6) and 4 ($t = -2.0189$, $df = 11$, p-value = 0.06855) at both timepoints were also discovered to be insignificant. The inconsistency of the intensity of the bands 1, 2, 3 and 4 across and within each treatment group led to them being eliminated as possible indicators for digestion rate.

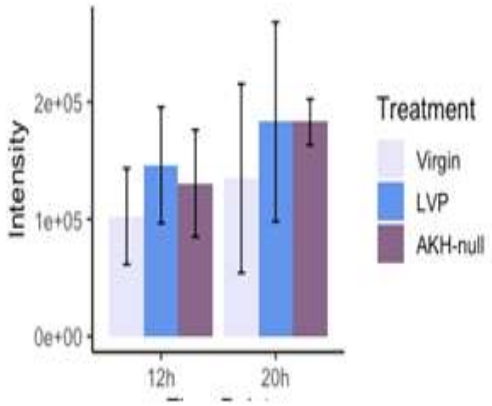

Figure 14: Bar graph of the intensities of band 4 of the various treatment groups at different time intervals (obtained from SDS-PAGE). The lavender coloured bar corresponds to the LVP virgin mosquitoes, the blue coloured bar corresponds to the LVP females mated to LVP males while the plum coloured bar corresponds to LVP females mated to AKH-null males. The error bars represent the standard deviation of the intensities in each treatment group. 12h and 20h represent the mosquitoes frozen 12hrs and 20hrs after feeding.

Western blots were tried as a method of visualizing the change in pattern of the bovine albumin serum present in the bloodmeal of mosquitoes over time. The methods used did not produce feasible results. A 1:100, 1:250, 1:500 and 1:1000 ratio of primary antibody to 1X casein (dissolved using TBST) was used to visualize the protein bands present in the antibody; however, the western blot images showed up empty. Through discussion with Dr. Dean Fraga (The College of Wooster, Wooster, OH), it was discovered that bovine albumin serum is usually difficult to detect in western blots; either a higher concentration of primary antibodies or a higher amount of blood sample should have been used in order for good sized protein bands to show up on the western blot.

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Table 3: Ratio of the Intensity of the bands present at 25kD and bands present at 75kD.

TREATMENT	Virgin	Virgin	LVP	LVP	AKH-null	AKH-null
Time Point	12hrs	20hrs	12hrs	20hrs	12hrs	20hrs
Figure 7						
Ratio of 25kD bands	1.68	0.65	0.77	0.66	1.20	0.67
Ratio of 75kD bands	1.09	1.00	1.84	1.36	1.22	1.14
Figure 8						
Ratio of 25kD bands	0.88	0.95	0.72	1.21	1.09	1.26
Ratio of 75kD bands	1.91	1.47	1.17	1.87	2.58	1.05
Figure 9						
Ratio of 25kD bands	2.50	0.70	0.56	0.58	0.47	1.15
Ratio of 75kD bands	1.41	1.24	1.13	1.09	1.07	1.05
Figure 10						
Ratio of 25kD bands	0.66	0.70	0.75	0.67	0.94	1.03
Ratio of 75kD bands	1.28	1.17	1.43	1.16	1.46	1.01

Note: 25kDbands are bands 1 and 2; 75kD bands are bands 3 and 4

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