Biological rhythms in *Aedes aegypti* mosquitoes

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ABSTRACT

Aedes aegypti mosquitoes are found globally and also act as the primary vector of Zika, dengue, and Chikungunya viruses, for which there are limited treatment options and no vaccines available. The use of insecticides as the main control strategy against diseases transmitted by this mosquito, is increasingly challenged by emerging resistance. Thus, there is a dire need for the development of novel approaches informed by an improved understanding of mosquito biology, to control mosquito populations and, ultimately, disease transmission. Rhythmic biological processes in mosquitoes help optimize resource exploitation by coordinating behaviors and physiology with fluctuating environmental conditions. Such synchronization enables organisms to adjust their physiology, metabolism, and behavior to predictable external cycles. In mosquitoes, circadian rhythmicity has been demonstrated in their biting and oviposition behavior, as well as their locomotor activity. However, little is known regarding how responses to long-range host-cues are modulated by the circadian system. Here we show that both antennal sensitivity and olfactory behavior are time-of-day and odor-specific in *Ae. aegypti* females. Global transcriptomic analysis in whole heads of *Ae. aegypti* females reveal chemosensory genes differentially expressed throughout the day, providing insight into the molecular mechanisms behind daily variations in olfactory sensitivity and behaviors. We additionally show an odor-induced activation of mosquito behavior. Mosquito locomotion and behavior are also mediated by physiological state, and activity decreases after blood-feeding. Since the central clock components have been shown in other organisms to be redox-sensitive, we explored the role that diet heme plays in mediating behavioral changes following blood ingestion using artificial blood diets. We found that the transcription of the timekeeping gene *period* is reduced in the head immediately after feeding on a meal containing hemoglobin, but peripheral *period* transcription is reduced throughout the course of digestion following ingestion of a protein meal independent of hemoglobin inclusion. Overall, our results show that *Ae. aegypti* behavioral rhythms mediated by rhythmic gene expression are plastic and susceptible to external host cues and host blood digestion. This work can be leveraged for future studies investigating mosquito host-seeking and blood digestion to identify novel targets for vector control.

Biological rhythms in *Aedes aegypti* mosquitoes

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GENERAL AUDIENCE ABSTRACT

Female mosquitoes rely on blood-feeding in order to produce eggs, but can unfortunately act as vectors of disease if they transmit pathogens when biting. Insecticides are currently our strongest main tool for controlling mosquito disease vectors such as *Aedes aegypti*, the yellow fever mosquito. However, increasing cases of insecticide resistance present new challenges in vector control, and new strategies to prevent vector-borne disease are needed. The *Ae. aegypti* mosquito is found globally and transmits Zika, dengue, and Chikungunya viruses, for which there are limited treatment options and no vaccines available. Mosquitoes exhibit rhythms in their gene expression and behaviors such as biting and activity patterns, in order to optimize energy efficiency and coordinate their biology and behaviors with daily fluctuations in the environment. However, it is unknown how their responses to human host odor cues are modulated by their central timekeeping system in the brain. Mosquitoes primarily find a human host via their sense of smell, or olfaction. Odor molecules in the air, emitted by humans, can be detected by mosquitoes' antennae. Here we show that both antennal sensitivity and behavioral responses to odors are time-of-day and odor-specific in *Ae. aegypti* females. We quantified gene transcripts in whole heads of *Ae. aegypti* females as a measure of gene expression, which revealed that genes involved in odor detection are expressed differently throughout the day, providing insight into the molecular mechanisms behind behavioral observations. We also show that mosquito behavior can be activated by odor exposure, and that their behavioral patterns can be influenced for multiple days following exposure. Mosquito behavior is also influenced by blood-feeding, which reduces mosquito activity and flight. Time-keeping genes in the fly brain have been shown to be sensitive to oxidative stress. Blood contains the protein hemoglobin, which can lead to oxidative stress when digested. Using artificial blood diets that allowed us to include or exclude hemoglobin in the meal, we found that the transcription of the timekeeping gene *period* is reduced in the head immediately after feeding on a diet containing hemoglobin, but is reduced in the rest of the body throughout the course of digestion following ingestion of a protein meal, whether hemoglobin was included or not. This work can be leveraged for future studies investigating mosquitoes' rhythms in host-seeking and blood digestion to identify new effective targets for vector control.

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help me laugh and letting me be an aunt to my amazing nephews and niece, Maverick, Maddox, and Aerowyn. I thank my sister, Renee Eilerts, for her unwavering support and love, always welcoming me in her home, and letting me crash her vacations.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
cat	Catalase
cDNA	Complementary DNA
Clk	Clock
Cry	Cryptochrome
Cyc	Cycle
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EAG	Electroantennography
for	Foraging
FPKM	Fragments per Kilobase per Million
GO	Gene ontology
GTP	Guanosine triphosphate
Hb	Hemoglobin
ho	Heme oxygenase
IR	Ionotropic receptor
LD	Light:dark
Mw	William’s mean
NMDS	Non-metric multidimensional scaling
noHb	No hemoglobin
npylr	Neuropeptide Y-like receptor I

OBP	Odorant binding protein
OR	Odorant receptor
Pdp1	PAR-domain protein 1
per	Period
PKG	cGMP-dependent protein kinase
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
snpf	Short neuropeptide F
Tim	Timeless
TPM	Transcripts per Kilobase per Million
Vri	Vrille
WGCNA	Weighted gene correlation network analysis
WHO	World Health Organization
ZT	Zeitgeber time

CHAPTER 1: INTRODUCTION

1.1 Biological rhythms

Rhythmic biological processes are observed in a wide variety of organisms, including insects, and optimize the allocation of resources by coordinating behaviors and physiology with the external environment (Meireles-Filho and Kyriacou, 2013). Daily and seasonal rhythms in external environmental cues (such as light or temperature) impose selective pressures on organisms to tune their physiological processes and behaviors to synchronize to such rhythms.

Organisms, therefore, evolved an internal biological clock in order to endogenously maintain rhythms with 24-hour periodicity, which can be entrained by environmental cues such as day-length and seasons change. While some rhythmic processes are directly driven by the environmental light/dark cycle and are no longer observable in the absence of external cues, other rhythms are endogenously sustained, even in the absence of external Zeitgeber (meaning *time giver* in German; *i.e.*, rhythmically occurring environmental cue). These rhythmic biological processes are qualified truly circadian when they fulfill three properties: 1) they are self-sustaining and free-running so that oscillations will continue with approximately 24-h periodicity even in the absence of external environmental cues; 2) they are able to synchronize or entrain to adjust the clock period to match environmental conditions; and 3) they are able to compensate for temperature and maintain 24-h periodicity (Meireles-Filho and Kyriacou, 2013; Sandrelli et al., 2008; Tomioka and Matsumoto, 2010).

Our understanding of the clock at cellular and molecular levels has been garnered from studies of model organisms such as the fruit fly, *Drosophila melanogaster*. Within the fly brain, approximately 150 circadian clock neurons function to control rhythmic behaviors (Allada and Chung, 2010; Nitabach and Taghert, 2008). The endogenous biological clock within clock neurons is primarily driven by two interlocked transcriptional and translational feedback loops (Figure 1.1). Transcriptional factor proteins CLOCK (CLK) and CYCLE (CYC) heterodimerize and bind to promoters of *period (per), timeless (tim), vrille (vri),* and *PAR-domain protein 1 (Pdp1*) genes to initiate their transcription. When PER and TIM proteins accumulate in the cell, they heterodimerize for transport into the nucleus, where they shut down their expression by binding and sequestering CLK and CYC proteins. Once PER and TIM expression levels are low enough, CLK and CYC are no longer inhibited and a new round of their transcription ensues. Clk transcription is regulated by VRI and PDP1 proteins, which compete for binding to the Clk promoter: VRI represses transcription while PDP1 activates transcription, resulting in the cyclic expression pattern of CLK (Allada and Chung, 2010; Cyran et al., 2003; Stanewsky, 2002).

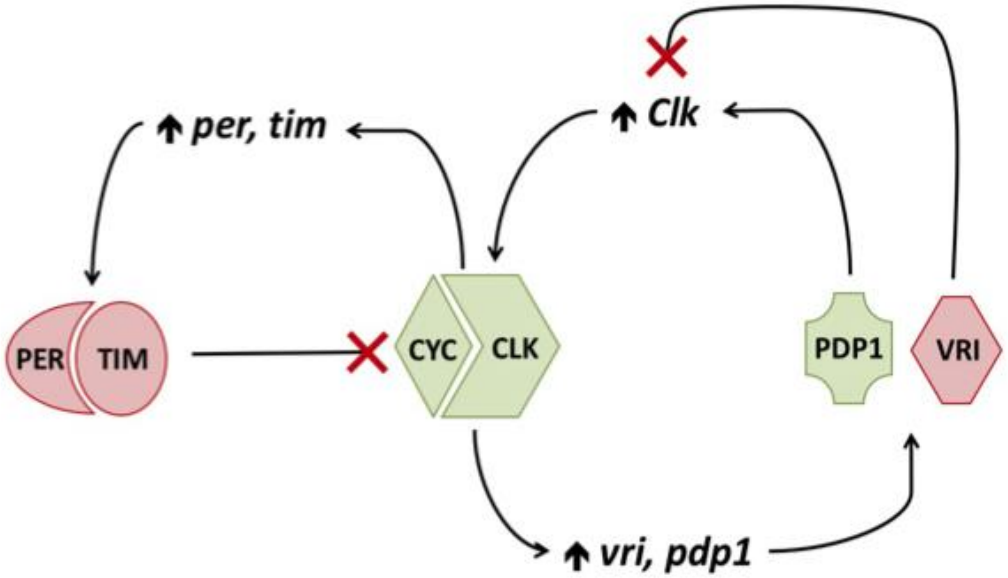

Figure 1.1. Circadian rhythm transcript factor feedback loops. CLK and CYC dimerize to activate expression of PER, TIM, VRI, and PDP1. PER and TIM

dimerization inhibits CLK/CYC. VRI and PDP1 regulate Clk transcription. CLK: Clock; CYC: Cycle; PDP1: PAR-domain protein 1; PER: Period; TIM: Timeless; VRI: Vrille; Green: activator; Red: repressor. Adapted from (Nitabach and Taghert, 2008).

Resetting of the clock by external light cues is achieved via the blue light sensitive flavoprotein Cryptochrome (CRY), which binds to TIM and initiates TIM degradation. In some insect species (*i.e.*, mosquitoes and moths), there are two cryptochrome genes, as is the case in mammals. CRY1 is a blue light photoreceptor responsible for TIM degradation, the mechanism of circadian photosynchronization. CRY2 is implicated as a negative regulator. In mammals, CRY2 is a light-independent inhibitor of CLOCK-BMAL1 (homologous to CLOCK-CYCLE in insects) (Gentile et al., 2009; Yuan et al., 2007).

Circadian outputs are rhythmic adaptations to specific environmental conditions that benefit survival and reproductive fitness. Sleep-wake cycles are one of the most obvious circadian outputs. Circadian clocks also modulate activity patterns to enhance survival by avoiding predators, exposure to environmental stressors such as UV radiation, avoiding competition with other species, or by enhancing their success of finding a mate or food (Paranjpe and Sharma, 2005; Patke et al., 2020). The clocks also regulate eclosion time in most insects to synchronize eclosion to times of day with higher humidity to prevent desiccation and enhance survival rates (Pittendrigh, 1954). Of increasing interest is also the role of internal clocks in maintaining metabolic rhythms (Wijnen and Young, 2006). For example, genes with functions related to detoxification, immune response, hormone production, and neuropeptide response have been shown to exhibit circadian regulation in flies (Lin et al., 2002; McDonald and Rosbash, 2001; Wijnen et al., 2006).

1.2 *Aedes aegypti* mosquitoes

Mosquitoes are not exempt from this rhythmic way of life. Mosquitoes are highly dependent on circadian rhythms to maintain several biological and physiological processes such as sugar and blood feeding, locomotor and flight activity, mating, and oviposition (Rund et al., 2016). Cyclic behavior is necessary for effective host-seeking in order to ensure that mosquitoes are active at a time of day conducive to finding an appropriate host. For both sugar and blood feeding, mosquitoes must align their efforts with the rhythms of their feeding targets. For example, plant nectar sources show rhythms in floral blooming and odor emission, as well as nectar secretion (Fenske and Imaizumi, 2016; Matile, 2006; Overland, 1960). Similarly, human hosts display daily rhythms in their presence inside or outside of their dwellings and whether they are awake and defensive or resting. To identify and locate these potential hosts, female mosquitoes rely primarily on the detection of olfactory signals. Previous studies have identified a variety of genes important for such host-seeking exhibiting circadian and diel rhythmic expression in mosquitoes. In *Anopheles gambiae* mosquitoes, a vector of malaria, it has also been shown that olfactory sensitivity to specific odorants varies in a rhythmic time-of-day dependent manner (Rund et al., 2011; Rund et al., 2013a; Rund et al., 2016). Beyond that, rhythms in mosquito olfactory behavior have been largely overlooked. This is a critical knowledge gap because mosquito bites result in disease transmission contributing to over 1 million deaths every year, making mosquitoes the deadliest animal in the world (WHO, 2018). While both male and female mosquitoes feed on nectar, only females require blood-feeding to obtain the proteins and nutrients necessary for egg production and development. When feeding on a host infected with a virus or a parasite, they too become infected and can later transmit the pathogen to a new host. *Ae. aegypti* mosquitoes are a domesticated and anthropophilic species, therefore they live and breed near humans (Takken and Verhulst, 2013). Feeding preferentially on humans, *Ae. aegypti* also have a tendency to bite multiple times per gonotrophic cycle and in their lifetime (Scott and Takken, 2012). Unfortunately, this contributes to their role as the primary vectors of dengue, chikungunya, yellow fever, and Zika viruses. However, for this species as well, olfactory rhythms remain to be fully characterized.

For efficient host-seeking, rhythmic mosquito behavior is modulated by several factors. Diel rhythms are directly driven by rhythmic environmental light cues such as sunrise or sunset, while circadian rhythms are controlled by endogenous biological clocks. Together, these rhythms enable mosquitoes to adjust their physiology, metabolism, and behavior to predictable environmental cycles to optimize resource allocation. For example, rhythmic patterns in locomotor activity, sleep-wake cycle behaviors, oviposition, and blood-feeding have been demonstrated in mosquitoes (Ajayi et al., 2020; Eilerts et al., 2018; Fritz et al., 2008; Fritz et al., 2014; Gentile et al., 2006; Rund et al., 2016).

1.3 Mosquito blood-feeding and metabolism

In addition to the possibility of being exposed to infectious pathogens in the blood meal, blood-feeding is a high-risk behavior for mosquitoes: once they land on a host to obtain a blood meal, they are exposed to potential harm from a defensive host and must cope with physiological stressors associated with the ingestion of blood itself. Not only do they ingest large volumes of a relatively hot fluid that causes heat stress (Benoit et al., 2011; Lahondère and Lazzari, 2012), but blood also contains high quantities of the iron-binding protein hemoglobin, which releases free iron and heme upon metabolic processing. While these are essential for many physiological processes, they can also be extremely toxic as they catalyze the production of reactive oxygen species (ROS) that can damage nucleic acids and proteins and lead to uncontrolled lipid peroxidation (Whiten et al., 2017). Because female reproductive success depends on blood-feeding, they have unique mechanisms for protection against damage associated with iron and heme-induced oxidative stress (Pereira et al., 2007; Tsujimoto et al., 2018).

As mosquito-host interactions are generally time-of-day dependent, protective mechanisms against such stressors have been suggested to be regulated by the circadian system (Balmert et al., 2014; Krishnan et al., 2008; Rund et al., 2016; Yang et al., 2010). However, *Aedes* mosquitoes are capable of opportunistic blood-feeding outside of their typical host-seeking time window (Christophers, 1960; Lehane, 2005) and can therefore be exposed to blood stressors at times where these protective mechanisms are not optimal. In this context, *Aedes* mosquitoes provide a unique opportunity to experimentally decouple circadian physiological rhythms and oxidative stress responses. Previous work in *Drosophila* established that oxidative stress management and molecular components of the biological clocks are involved in a bidirectional interaction; furthermore, behaviors that are normally rhythmic in flies rapidly became arrhythmic in mutant flies with increased sensitivity to oxidative stress (Hardeland et al., 2003; Krishnan et al., 2008; Mandilaras and Missirlis, 2012; Zheng et al., 2007). While *Drosophila* is an excellent genetic model for characterizing the underlying molecular mechanisms of such a phenomenon and their potential involvement in circadian processes, female mosquitoes provide a unique opportunity to dive deeper into these mechanisms as they ingest several iron-rich meals during their adult life. Mosquito blood-feeding behavior is principal to their role as disease vectors and despite the inherent need for effective iron metabolism strategies in blood-feeding arthropods, the interplay between the biological clock and oxidative stress mechanisms remains to be defined.

Previous research in *Aedes* mosquitoes has shown that ingestion of the blood meal, which predominantly occurs at specific times-of-day (Trpis et al., 1973), causes ROS production in the midgut epithelium to decrease, ultimately leading to increased bacterial proliferation (Oliveira et al., 2011). It has been demonstrated that viral infection can alter vector behavior (Grimstad et al., 1980; Jackson et al., 2012; Maciel-de-Freitas et al., 2013; Sim et al., 2012) and a number of studies have reported up- or down-regulation of specific genes that maximize transmission of the pathogen, sometimes with a fitness cost to the vector (Shrinet et al., 2018). Integrated -omics data from multiple databases and metabolomic profiling of *Aedes* mosquitoes infected with dengue and/or chikungunya viruses show broad significant changes in expression to an extensive oxidative stress-related pathway network following viral infection (Shrinet et al., 2018), indicating that these pathways work in a harmonized manner to regulate oxidative stress upon arboviral infections. Redox equilibrium is critical for cellular processes and influences pathogen infection. For example, in *Ae. aegypti*, high levels of oxidative stress have been shown to inhibit pathogen infection (Molina-Cruz et al., 2008) and oxidative stress has been shown to increase in insect cells upon viral infection (Chen et al., 2011). In summary, oxidative stress management in mosquitoes is inherently complex, as there are multiple and often conflicting selective pressures for adaptive strategies for redox homeostasis.

1.4 Outstanding questions and dissertation goals

Mosquito-host interactions are central to mosquitoes' role as effective disease vectors. Mosquitoes rely primarily on odorant perception in order to efficiently seek out a human host; once a mosquito successfully finds and bites a human host, they must digest the blood meal for reproduction. However, how time of day influences molecular and physiological processes that underlie rhythms in host-seeking remain poorly understood. In addition, the role of the oxidative stress induced by blood-feeding in affecting the activity pattern of mosquitoes has been overlooked.

In this context, the objectives of the present dissertation were to:

1. Quantify the daily rhythms in *Ae. aegypti* olfactory behavior and sensitivity (Chapter 2). In this chapter, we quantified olfactory behavior and sensitivity to a small panel of host and plant odors throughout the day in female *Ae. aegypti* mosquitoes.
2. Establish the molecular basis of olfactory rhythms (Chapter 3). We performed transcriptomic analyses of female *Ae. aegypti* head tissue to evaluate daily rhythms in gene expression in the brain and sensory appendages.
3. Determine the plasticity of olfactory-mediated host-seeking rhythms (Chapter 4). We developed and performed behavioral experiments to

quantify the robustness of diurnal host-seeking behaviors, and evaluate entrainment to host odor cues.

4. Identify the impact of diet hemoglobin on mosquito behavioral rhythms (Chapter 5). We fed *Ae. aegypti* female mosquitoes one of four test diets and further examined to examine how diet hemoglobin impacts behavioral rhythms and transcript levels throughout digestion.

CHAPTER 2: ODOR-SPECIFIC DAILY RHYTHMS IN THE OLFACTORY SENSITIVITY AND BEHAVIOR OF *AEDES AEGYPTI* MOSQUITOES

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2.1 Abstract

Many biological processes and behaviors in mosquitoes display rhythmic patterns, allowing for fine tuning to cyclic environmental conditions. In mosquitoes, vector-host interactions are primarily mediated by olfactory signals. Previous studies have established that, in the malaria vector *Anopheles gambiae*, rhythmic expression of odorant binding proteins and takeout proteins in the antenna resulted in a corresponding rhythm in olfactory sensitivity to relevant host odors. However, it remained unclear how rhythms observed in olfactory sensitivity affect or explain rhythms in behavioral output, which ultimately impacts disease transmission. In order to address this knowledge gap, we quantified and compared patterns in locomotor activity, olfactory sensitivity, and olfactory behaviors in adult female *Aedes aegypti* mosquitoes. Here, we demonstrate an odorant-specific modulation of olfactory sensitivity in *Ae. aegypti*, decoupled from rhythms in olfactory behavior. Additionally, behavioral assays performed herein represent the first evidence of a time-dependence of the olfactory activation of behavior in *Ae. aegypti* mosquitoes. Results suggest that olfactory behavior of *Aedes* mosquitoes is modulated at both the peripheral (antenna) and central levels. As such, this work serves as a foundation for future studies aimed at further understanding the neural and molecular mechanisms underlying behavioral plasticity.

Keywords: daily rhythms; biological rhythms; mosquitoes; vectors; olfaction; electrophysiology; behavior

2.2 Introduction

Rhythmic biological processes are observed in a wide variety of organisms and optimize allocation of resources by coordinating behaviors and physiology with rhythmically fluctuating external conditions (e.g., cyclical changes in light, humidity, temperature, rhythmic activity patterns of prey and predators). Diel rhythms are directly driven by rhythmic environmental cues and differ from circadian rhythms in the sense that they are not controlled by endogenous biological clocks. Diel rhythms are therefore not sustained in the absence of rhythmic external cues. Together, diel and circadian rhythms enable organisms to adjust their physiology, metabolism, and behavior to predictable environmental cycles.

Among disease vector insects, rhythmic patterns of locomotor activity (Jones et al., 1967; Peterson, 1980; Rowland, 1989; Taylor and Jones, 1969), oviposition (Sumba et al., 2004) (Fritz et al., 2008) (Chadee and Ritchie, 2010), blood-feeding (Fritz et al., 2014), and olfactory behavior have been demonstrated in several species (Meireles-Filho and Kyriacou, 2013). In most cases, they enable insects to synchronize their daily activity, and in particular their food-seeking behavior during periods when their hosts are resting, and are therefore the least able to defend themselves (Advances in insect physiology, 2009; Barrozo et al., 2004). From a sensory perspective, these vector-host interactions are mediated to a large extent by olfactory signals. Volatile chemicals indeed serve as cues that allow these insects to detect, identify, and locate their hosts from distances at which they remain safe from the threat of defensive behaviors. Interestingly, and conversely to what had been suggested in other insects (e.g., (Krishnan et al., 1999; Page and Koelling, 2003; Zhou et al., 2005)), disease vectors do not show a modulation in the overall sensitivity of their antennae for all chemical stimuli at the same time, but rather an activity and time-of-day dependent tuning of their olfactory sensitivity. For example, work on the malaria vector mosquito, *Anopheles gambiae*, showed that rhythms in antennal abundance of odorant binding proteins (OBP) lead to daily rhythms in olfactory sensitivity to major host-related chemicals, but not all rhythms are maintained in dark-treated mosquitoes (Rund et al., 2013a). In triatomine bugs, behavioral studies showed that the responsiveness to carbon dioxide is controlled by endogenous circadian clocks, while attraction to aggregation pheromones depends on environmental signals (Barrozo et al., 2004; Bodin et al., 2008). While these studies provide evidence that endogenous clocks and exogenous cycles act together to adaptively modulate sensory responses, whether rhythms in olfactory sensitivity are sufficient to fully explain the temporal modulation of the behavioral output remains unclear.

The yellow fever mosquito, *Aedes aegypti*, is an ideal experimental model to explore the relationship between rhythms of olfactory sensitivity and temporal modulation of behavior as behavioral rhythms and bimodal activity patterns are well-documented, and biting behaviors are mainly observed during the second activity peak (Taylor and Jones, 1969). This additionally suggests a selective daily modulation of the olfactory behavior. However, the comparison in rhythmic sensitivity patterns between *Aedes* and *Anopheles* mosquitoes remains to be made. To close this knowledge gap, in the present study, we examined the relationship between daily variations in antennal sensitivity and behavioral responses in *Ae. aegypti* mosquitoes. Both plant- and host-related odors were tested during the peaks and troughs of their locomotor activity patterns. Finally, independently quantifying the flight activity patterns of the mosquitoes allowed us to decouple spontaneous activity from odor-evoked activation of flight.

2.3 Materials and Methods

Mosquitoes

Two strains of *Ae. aegypti* mosquitoes were used for the experiments: Liverpool (actometer experiments; LVP-IB12, MR4, ATCC®, Manassas, VA, USA) and Rockefeller (all other experiments; MR-734, MR4, ATCC®, Manassas, VA, USA). Survey of the literature did not reveal any difference in the spontaneous locomotor activity of various strains of *Aedes aegypti* mosquitoes (Berry et al., 1986; Gentile et al., 2006; Taylor and Jones, 1969), discarding potential bias associated with strain differences. In addition, rhythms in peripheral sensitivity and olfactory behavior were analyzed within the same strain (Rockefeller) to prevent any bias being introduced. Larvae were raised in a 26 × 35 × 4 cm covered tray filled with ~1 cm of deionized water, at a density of approximately 200 larvae per tray. They were maintained under light:dark (LD) cycles of 12 h:12 h at 25 °C and 60 ± 10% humidity. Larvae were fed a daily diet of Hikari Tropic First Bites (Petco, San Diego, CA, USA). Pupae were isolated on the day of pupation and placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA—1425, 1425DG). These containers were then transferred to opaque plastic containers (Rubbermaid Brute boxes, 71 × 44 × 38 cm) at least three days prior to experiments to allow mosquitoes to synchronize to a light cycle aligned with the experimenters' work hours for one of the four tested Zeitgeber time (ZT) periods (0–2, 5–7, 10–12, 17–19) on a 12 h:12 h LD cycle where lights-on takes place at ZT 0 and lights-off at ZT 12. In all cases, mosquitoes tested at ZT

0–2 were taken from their rearing containers only after the onset of the lights, and all experiments conducted at ZT 17–19 were performed under red light illumination. For all experiments except actometer experiments, 6 ± 1 day-old adult female mosquitoes were used only once.

Activity Analysis

Locomotor and flight activity of female mosquitoes were recorded using a locomotor activity monitor (Trikinetics LAM25, Waltham, MA, USA). The LAM25 system consists of a vertical printed circuit board (PCB) with 32 openings, each equipped with 3 sets of infrared emitters and detectors. One to two days after emergence, individual mosquitoes were placed in glass cylindrical tubes with access to 10% sucrose at one end (Figure 2.1a). Mosquitoes were maintained in the tubes for a duration of 8 days. The tubes were positioned through the openings on the panel so that the infrared beams were located at the approximate center of each tube. Daily locomotion was recorded as the number of beam crossing per 30 min intervals using the DAMSystem3 Software (Trikinetics, Waltham, MA, USA). All recordings occurred in a light-proof enclosure with its own lighting system, which consisted of a light-emitting diode (LED) light (800 Lumen, Philips, Amsterdam, The Netherlands) timed to a 12 h:12 h LD cycle. Data analyses were performed on locomotor activity for a total of 94 mosquitoes and over either 4 or 5 consecutive days starting on the third instance of ZT 0 (i.e., on the third day), in order to ensure mosquitoes were appropriately entrained to the LD cycle. Individuals that were found dead at the end of the experiments were discarded from calculations after the last timepoint being recorded as active.

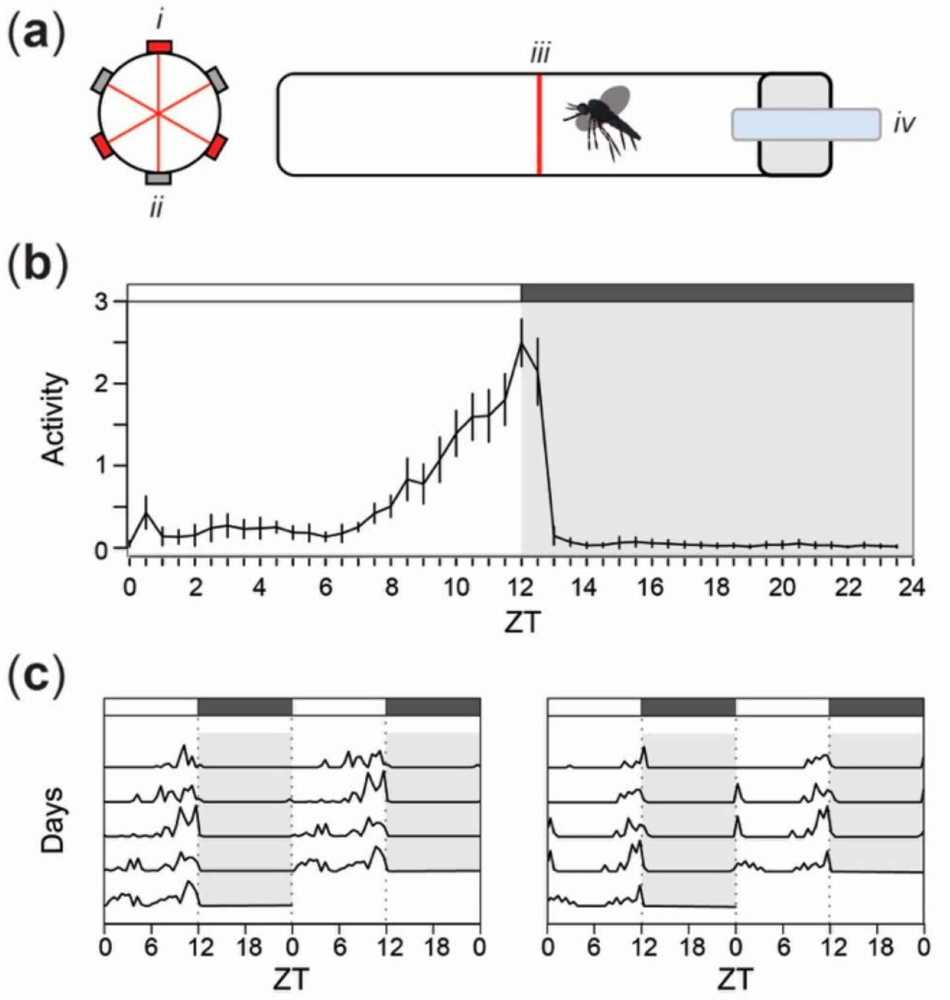

Figure 2.1. Locomotor and flight activity recordings in adult female *Ae. aegypti* mosquitoes show a bimodal pattern of activity, peaking in the last hours of the photophase. (a) Schematic representation of the locomotor activity monitoring system (Trikinetics LAM25, Waltham, MA, USA) showing a cylindrical glass tube containing an individual mosquito as viewed from the tube opening (left) and from the side (right). (i) Infrared laser emitters and (ii) detectors (iii) bisect each tube, which is enclosed with a (iv) sugar reservoir for constant access to 10% sucrose. (b) Mean locomotor activity pattern of 94 *Ae. aegypti* females in 12 h:12 h light-dark (LD) cycle. Values on the x-axis correspond to Zeitgeber time (ZT). Error bars represent the standard error around the mean. (c)

Double-plotted actograms illustrating representative locomotor activity in two individual mosquitoes in 12 h:12 h LD cycles for 5 consecutive days, following a 48-hour period of light entrainment. Gray background and boxes above the actograms indicate the scotophase (i.e., dark period of the cycle) while white background and boxes depict the photophase (i.e., light-on phase).

Olfactory Sensitivity

Electroantennography was used to quantify the olfactory sensitivity of 10–12 mosquitoes tested at four different times (ZT 0–2, 5–7, 10–12, and 17–19), corresponding to dawn, midday, dusk, and midnight for the insects. Mosquitoes tested at ZT 17–19 were prepared in functional darkness under red light, not visible by the mosquitoes (Muir et al., 1992), and tested in dark conditions. Individuals tested were immobilized on ice before their head was excised with surgical micro-scissors and mounted on an indifferent (i.e., reference) glass electrode. The recording electrode accommodated the two antennae of the excised head after the tips of the antennae were clipped to provide a better contact (Figure 2.2a). Both electrodes consisted of oxidized silver wires inserted into drawn-out glass capillary tubes filled with saline solution (Beyenbach and Masia, 2002). The signal from the electrodes was amplified by a microelectrode AC amplifier (Model 1800; A-M Systems, Sequim, WA, USA) and was filtered with a HumBug noise eliminator (Quest Scientific, North Vancouver, BC, Canada). The signal was then recorded using a winEDR Strathclyde Electrophysiology Data Recorder V3.8.0 (University of Strathclyde, Glasgow, UK).

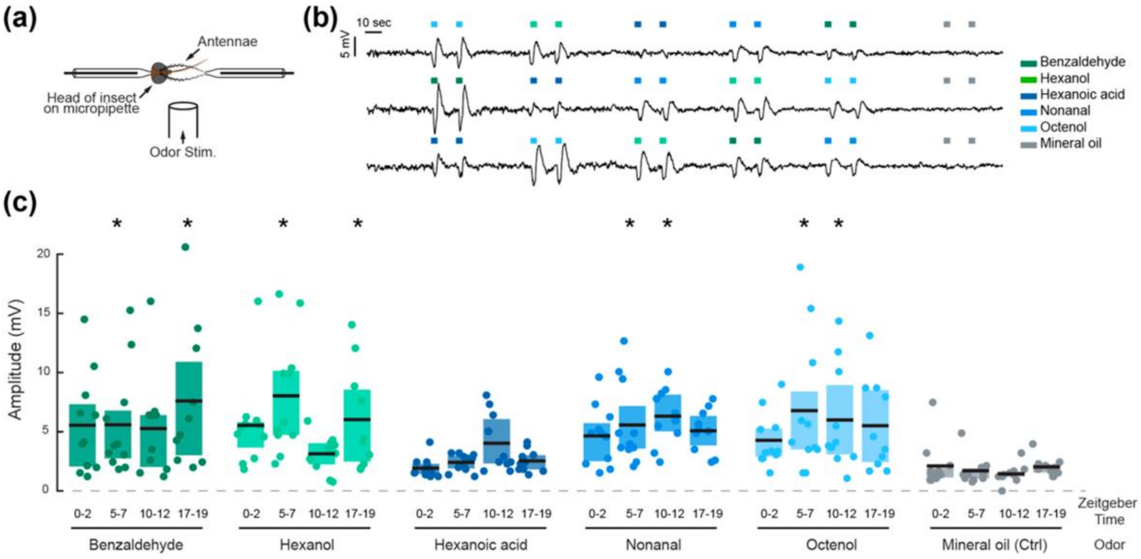

Figure 2.2. Olfactory sensitivity is time and odorant specific in adult *Ae. aegypti* females. (a) Schematic of the electrophysiological preparation for electroantennogram recordings. (b) Three representative traces depicting typical voltage deflection after olfactory stimulation of two consecutive pulses of five odorants, delivered in a randomized order. (c) Mean amplitude of the electrophysiological responses to the five odorants and mineral oil control at four different times of day. Each dot represents the response of an individual mosquito, black lines indicate the mean response at each time point, and shaded areas represent the first and last quartiles. Asterisks denote responses that are significantly different from the respective mineral oil control (Tukey post-hoc test, $p < 0.05$).

Volatiles tested were benzaldehyde, nonanal, hexanol, hexanoic acid, and 1-octen-3-ol (octenol). Chemicals were obtained from Sigma-Aldrich and were prepared by dilution (1:100) in mineral oil (M8410–1L) for final concentrations of: 98 mM benzaldehyde (≥99.5%, 418099-100ML); 58 mM nonanal (95%, N30803-25G); 80 mM hexanol (Anhydrous, ≥99%, 471402-100ML); 80 mM hexanoic acid (≥99%, 153745-100G); and 65mM octenol (98%, O5284-25G). The rationale behind this choice was to test plant- (benzaldehyde and hexanol) and host-emitted compounds (hexanoic acid, octenol), at concentrations that are not limiting factors for quantifying mosquitoes' olfactory sensitivity. It is worth noting that nonanal can be found in the headspace of both plants and animals (Curran et al., 2005; Witzgall et al., 2005). Pure mineral oil was used as a control. The stimuli were delivered by pipetting 40 microliters of each odor onto a piece of Whatman filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) in a respective glass syringe. The tip of the syringe was then inserted into a glass tube delivering a constant air flow (30 cm·s^{-1}) from a medical air tank (Praxair, Inc., Danbury, CT, USA), the end opening of which was 12.3 mm in diameter and positioned about 5 mm from the mosquito head. The tip of the syringe was positioned 9.12 cm upstream of the opening. For each odor, two 2-second long pulses were delivered (2.3 cm·s−1) with a 10 second inter-pulse interval. In other words, the odorant concentrations were therefore further diluted ~13 times. Odor pulses were triggered by a 3-way solenoid valve (The Lee Company, Westbrook, CT, USA) controlled by a custom-written Matlab script (The MathWorks Inc., Natick, MA, USA). All five odors were delivered in a series to each mosquito head, and the order was randomized in Matlab. Mineral oil was used as a control and delivered before and after the five odors. Preparations that displayed background levels below one standard deviation above the mean background level of our sampled population (indicating proper electrophysiological montage) and for which no significant responses (i.e., not different from responses to mineral oil) were observed, were considered as non-responsive and discarded from the analysis. For each time point, 10–11 responsive mosquitoes were used for the analysis.

Olfactory Behavior

An enclosed Y-maze olfactometer (Vinauger et al., 2018) was used to quantify the behavioral responses of mosquitoes to the same plant- and host-related chemical compounds used in the electrophysiology experiments, at the same concentrations (i.e., benzaldehyde, nonanal, hexanol, hexanoic acid, and octenol). The olfactometer consisted of custom-cut acrylic with two choice arms and one entrance arm connected to a central chamber (100 cm long, 10 cm internal diameter, choice arms positioned at a 120° angle, Figure 2.3a). A constant airflow was generated by two fans (Rosewill, Los Angeles, CA, USA, air speed ~30 $\mathrm{cm{\cdot}s^{-1}}$) mounted at the end of the choice arms and was filtered through a series of activated charcoal filters and honeycomb mesh (10 cm long) to create a contaminant-free laminar airflow. The olfactory stimuli were delivered through divided circuits of polytetrafluoroethylene (PTFE) tubing (McMaster-Carr, Elmhurst, IL, USA) and charcoal-filtered (~3 $\mathrm{cm{\cdot}s^{-1}}$), further diluting the odorant concentrations 10 times. The air was then passed through scintillation vials containing either the tested odor or the control solution (mineral oil). Each respective line was attached to the distal end of the corresponding choice arm through small holes in each of the arms of the Y-maze. To avoid environmental bias, the position of the stimulus and control currents were randomly exchanged in the olfactometer arms between experiments. Statistical analysis did not reveal any preference for the left or right side of the olfactometer ($n = 16$; Binomial Exact test: $p = 0.59$) when mosquitoes were tested against two clean (i.e., mineral oil laden) air currents at ZT 10–12. All experiments were performed in a well-ventilated chamber at a constant temperature (26 ± 2°C) and relative humidity (50–70%). For each odor and time point, 16 < n < 23 mosquitoes made a choice and were taken into account for preference analysis.

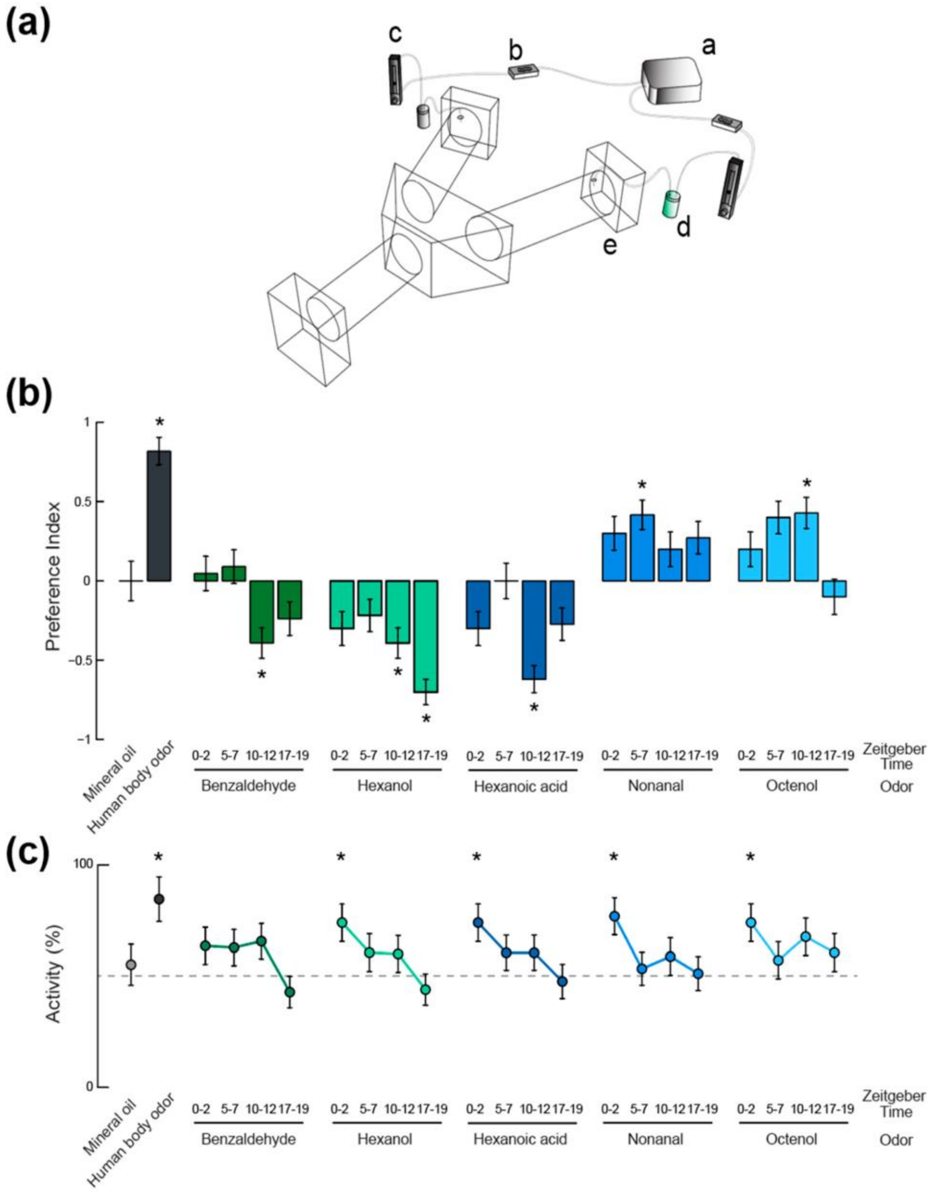

Figure 2.3. Time-of-day and odorant-specific olfactory activation and behavioral responses of *Ae. aegypti* adult female mosquitoes. (a) Schematic

representation of the Y-maze olfactometer used in behavioral assays. (i) air pump; (ii) charcoal filter; (iii) flowmeter; (iv) scintillation vial containing either the odorant stimulus or the solvent only; (v) delivery of the odor laden air into the choice arms of the olfactometer. Mosquitoes are released into the entrance arm and fly upwind where they are forced to make a decision between the two choice arms, each delivering a different odor stimulus. (b) Mosquito odor preference represented as a preference index. Asterisks denote behavioral responses that were significantly different from chance (Binomial Exact test, $p < 0.05$). (c) Proportion of activity (i.e., proportion of mosquitoes making an active choice between the two choice arms of the olfactometer) under different olfactory contexts and at four different times of day. Asterisks denote proportions that were significantly different from activity levels in absence of odor (55%; Binomial Exact test, $p < 0.05$). In (b,c), error bars represent the standard error to the mean.

Data Analysis

Activity data generated with the actometer, electrophysiological data, and binary data collected in the olfactometer were analyzed using R software (version 3.4.2 (R Development Core Team, 2020)). Activity data was analyzed by calculating the William’s Mean (Mw), a modified geometric mean to accommodate datasets with zero values (Haddow, 1960), of the activity for all mosquitoes monitored. Activity data for individuals observed dead at the end of the experiment were excluded following the last recorded time of activity. A strong “startle response” in the activity was observed at the lights-off, similarly to observations made by others (Gentile et al., 2006; Lima-Camara et al., 2011). Activity data recorded at this time were excluded and instead interpolated by calculating the arithmetic mean between activity values recorded immediately before and after lights-off (Gentile et al., 2006). This was done in an effort to avoid representing artificially heightened activity due to the abrupt lighting change at lights-off. Instead, this exclusion represents the locomotor activity at this time in a manner that is more likely representative of *Ae. aegypti* activity in nature occurring at sunset. The cyclicity of the activity of individual mosquitoes was tested by means of Fisher's Exact G-test, based on the null hypothesis of Gaussian white noise against the alternative of an added periodic component of unspecified frequency (Fisher, 1997).

Trigger-averaged electrophysiological data were analyzed for each odor stimulus and time point. The amplitude of the odor-induced voltage deflection were extracted and used for the analysis (ANOVA and Tukey multiple comparisons of means). Binary data obtained in the olfactometer were compared to chance by means of the Binomial Exact test ($\alpha = 0.05$).

2.4 Results

The overall pattern of locomotor activity of female *Ae. aegypti* under a 12 h:12 h LD cycle was consistent with previously published observations (e.g., (Gentile et al., 2006; Taylor and Jones, 1969)). The mean daily activity of 94 individuals shows a bimodal diurnal pattern of locomotor activity (Figure 2.1b). A first slight increase in average activity levels was observed within the first two hours after lights-on, followed by a period of low activity until approximately four hours prior to lights-off when a second increase in activity occurred. Locomotor activity reached a peak at approximately ZT 12 and immediately decreased dramatically within the first hour after lights-off. During the scotophase (i.e., the period of darkness) that follows (i.e., ZT 13–24), we observed minimal levels of activity. Of the 94 tested females, 71.3% displayed significantly rhythmic activity patterns (Fisher's Exact G-test, $p < 0.05$), similar to the two representative individual actograms depicted in Figure 2.1c.

The peaks and troughs of locomotor activity were selected as Zeitgeber times (ZT) for testing the peripheral olfactory sensitivity of the mosquitoes. The responses to five odorant volatiles (two aldehydes, two alcohols, and a carboxylic acid), were therefore tested at ZT 0–2, 5–7, 10–12, and 17–19 (Figure 2.2). The amplitudes of the odor-evoked responses were significantly influenced by the odorant stimuli (ANOVA; F6, 557, $p < 0.001$), the time of day (F3, 557, $p = 0.044$) and the interaction between the odorant and time of day (F18, 557, $p < 0.001$; Table 2.1). All odorants except hexanoic acid elicited significant electrophysiological responses compared to the mineral oil control pulses (Tukey post-hoc test; $p < 0.05$), but the patterns of daily olfactory sensitivity were odorant specific. Responses to octenol, nonanal, and hexanoic acid did not vary significantly throughout the day, while responses to benzaldehyde and hexanol significantly varied as a function of time (pairwise comparisons, Tukey post-hoc test, $p < 0.05$). The mosquitoes were most sensitive to benzaldehyde during the 17–19 h, to hexanoic acid during the 10–12 h, and to hexanol during the 5–7 and 17–19 h time periods. Interestingly, the proportion of responsive preparations (i.e., preparations that displayed at least one response significantly higher than baseline responses to mineral oil) followed the same trend as the locomotor activity rhythm, with 91.6 ± 4.8% of preparations responsive during the photophase, and 58.8% of preparations responsive during the scotophase ($n = 35$ and $n = 17$

respectively). The non-responsive individuals also displayed larger background noise levels.

Table 2.1. Analysis of variance (ANOVA) reveals that odorant sensitivity of female *Ae. aegypti* mosquitoes is significantly modulated by the odorant stimulus, the time of day, and the interaction between these two factors. **Df**: Degrees of Freedom; **Sum Sq**: sums of squares; **Mean Sq**: mean of squares; **F value**: values of the F statistic; **Pr(<F)**: the probability of observing an F ratio greater than the corresponding F value.

	Df	**Sum Sq**	**Mean Sq**	**F Value**	**Pr(>F)**
Odor	6	1419	236.46	24.711	$<2 \times 10^{-16}$
Time of Day	3	78	26.05	2.722	0.0437
Odor:Time of Day	18	435	24.15	2.524	0.0005
Residuals	557	5330	9.57		

In order to test how these daily variations in olfactory sensitivity affect the behavioral responses of the mosquitoes, a Y-maze olfactometer was used to examine their olfactory behavior (Figure 2.3b). A neutral control, for which a choice between two clean air currents was given, showed no bias in the experimental set up as mosquitoes oriented randomly between the two arms (n = 16; Binomial Exact test, p > 0.05). A positive control, using human feet odor extract, confirmed the suitability of the olfactometer to reveal oriented behaviors (n = 11; 90.9% attraction, Binomial Exact test: p = 0.011). The host-emitted aldehyde nonanal, for which no peripheral modulation of the olfactory sensitivity was observed, elicited low levels of attraction compared to our positive control.

Although no significant difference was observed between responses at different time points, only the ZT 5–7 tests induced a response that was significantly different from chance (i.e., a significant attraction; n = 24; 70.8% attraction, Binomial Exact test: p = 0.019). The other compound that did not show any response variation at the periphery, octenol, did elicit significantly different levels of behavioral response throughout the day. Only the responses to octenol at ZT 5–7 and 10–12 were either significantly different from chance or marginally significant (n = 20 and n = 21; 70% and 71.4% attraction; Binomial Exact test: p = 0.057 and p = 0.039, respectively). These levels of attraction were both significantly different from responses quantified during the scotophase (n = 20; Binomial Exact test: p = 0.021 and p = 0.013, respectively). For benzaldehyde and hexanol, the strongest behavioral responses were aversive (ZT 10–12, n = 23, 69.6% aversion, Binomial Exact test: p = 0.046 for benzaldehyde; ZT 10–12 and ZT 17–19, n = 23 and n = 20, 69.5% and 85% aversion, Binomial Exact test: p = 0.046 and p = 0.001, respectively, for hexanol) and were not observed in time windows during which the olfactory sensitivity was maximal for these chemicals (i.e., ZT 17–19 for benzaldehyde and ZT 5–7 for hexanol). Finally, responses to hexanoic acid were strongest during the maximum sensitivity window (ZT 10–12, n = 21, 81.0% aversion, Binomial Exact test: p = 0.004). Interestingly, all the other time windows showed responses that were not different from chance (20 < n < 23, Binomial Exact test: p > 0.05).

The activity levels in the olfactometer (i.e., the proportions of mosquitoes initiating flight and making a choice between one of the two decision arms), were consistent across odorant stimuli (Figure 2.3c). When compared to activity levels observed in the absence of odorant stimulus (55.1% of active mosquitoes; neutral control; N [i.e. n active mosquitoes

+ non-active individuals] = 29), the human feet extract (84.6% activity; Binomial Exact test: p = 0.027; N = 13), hexanol at ZT 0–2 (74.1% activity; Binomial Exact test: p = 0.033; N = 27), nonanal at ZT 0–2 (76.9% activity; Binomial Exact test: p = 0.018; N = 26), and octenol at ZT 0–2 (74% activity; Binomial Exact test: p = 0.034; N = 27) elicited significantly higher levels of flight initiations and decisions. Although not statistically significant, experiments conducted during mosquitoes' night times (i.e., ZT 17–19) led to fewer mosquitoes making a choice in the olfactometer than for the neutral control (33 < N < 50; Binomial Exact test: p > 0.05). The lowest level of activity observed being in response to benzaldehyde at ZT 17–19 (42.8% of activity; Binomial Exact test: p = 0.059; N = 49).

2.5 Discussion

In this study, we investigated the interplay between locomotor activity, olfactory sensitivity, and olfactory behavior in the disease vector mosquito *Ae. aegypti*. Three distinct questions were addressed in the present work: at the peripheral (i.e., antennal) level, does the sensitivity to odorant volatiles vary throughout the day? How does this daily rhythm in sensitivity translate into behavioral outputs? Are behavioral responses to odors limited by locomotor activity rhythms?

In the first part of this work, we confirmed the locomotor activity patterns observed by others in previous studies (Gentile et al., 2006; Taylor and Jones, 1969), with a small activity peak in the first hours of the photophase and a larger peak in the last hours of the photophase. The locomotor activity is reduced between these peaks, both in the photophase and the scotophase. Therefore, the peak and trough time windows were selected for the rest of the study to provide a baseline for comparing olfactory sensitivity and behavior at equally spaced times. Our next step was to use electroantennogram (EAG) analysis to quantify the olfactory responses induced by host- and plant-derived odorant chemicals. We purposely used concentrations within the same order of magnitude and above concentrations that mosquitoes would experience in nature (Hall et al., 1984; Jhumur et al., 2008; Syed and Leal, 2009). This allowed meaningful cross comparisons between odorants, and ensured that low responses observed in the electroantennogram recordings were not due to low concentrations, but actually indicated low sensitivity to that odor. In contrast to work conducted in the malaria vector *Anopheles gambiae*, in which daily patterns of olfactory sensitivity were conserved across most odorant chemicals (Rund et al., 2013a), here we observed an odorant specific modulation of the sensitivity. The level of peripheral sensitivity has been hypothesized to be mostly driven by rhythms in the expression patterns of Odorant Binding Proteins (OBPs) and takeout proteins (Rund et al., 2013a). This hypothesis largely relies on the fact that carboxylic acids (e.g., hexanoic acid and lactic acid), which are hydrophilic host odor constituents and, therefore, do not require OBPs for detection, are detected similarly at different times of day in *An. gambiae* and *Culex pipiens (Bowen, 1992; Rund et al., 2013a)*. However, here we observed that, although not significant, sensitivity to hexanoic acid also seemed to be time-of-day dependent, suggesting that, in *Aedes* mosquitoes, other regulatory processes might be at play.

Another particularity of *Ae. aegypti* mosquitoes is that, conversely to the nocturnal species mentioned above, these diurnal mosquitoes display a bimodal activity pattern (Figure 2.1; (Taylor and Jones, 1969)). While in the presence of available human hosts, *Ae. aegypti* females were observed in equal numbers at both activity peaks in landing assays conducted in the field (Chadee, 1988; Trpis et al., 1973), other behaviors, such as the oviposition behavior, are limited to the second activity peak (Gomes et al., 2006). The odorant-specificity in sensitivity rhythms that we observed is therefore not surprising, as some chemical compounds might be used by the mosquitoes in specific behavioral and temporal contexts. Benzaldehyde and hexanol are plant-associated odors and it is understandable that sensitivity to these odors would peak in times that are not associated with host-feeding. Additionally, our behavioral results show that *Aedes* mosquitoes are more repelled by these odors in the late hours of the day (i.e., ZT 10–12 and ZT 17–19), implying that they are not likely to be sugar-feeding at these times, when they are often observed to be host-seeking. Alternatively, mosquitoes showed higher antennal sensitivity to host-related odors (in particular nonanal and octenol) in the middle and end of the photophase (ZT 5–7 and 10–12), which is in line with their peak host-seeking times during the day. Mosquitoes showed significant attraction to nonanal in the middle of the day (ZT 5–7) and octenol near the end of the day (ZT 10–12), further indicating that olfactory processes constrain mosquitoes to primarily be host-seeking as opposed to sugar-feeding at these times. The high sensitivity and behavioral responses to host odors at midday, when only low levels of spontaneous locomotor activity are otherwise observed, suggest that these anthropophilic mosquitoes are capable of being activated by host kairomones and respond to opportunistic host encounters.

Another example of odor-specific rhythm is provided by studies performed in triatomine bugs, which also display a bimodal activity pattern. They were observed to seek for a host to bite in the beginning of the night and for a refuge to hide at dawn (Advances in insect physiology, 2009). More specifically, behavioral experiments showed that these insects respond to CO_2 only during the first hours of the night and are attracted to refuge-associated aggregation pheromones only at sunrise (Barrozo and Lazzari, 2004). Furthermore, analysis revealed that the circadian system is responsible for the modulation of the response to CO_2, whereas the responsiveness to aggregation pheromones is driven directly by environmental factors (Bodin et al., 2008). Rhythmic pheromone detection has been extensively studied in other insects, such as cockroaches (Schendzielorz et al., 2012; Zhukovskaya, 2008) and moths (Flecke et al., 2006; Merlin et al., 2007; Rosén et al., 2003; Schendzielorz et al., 2015). Further, the central circadian system has been established to modulate olfactory sensitivity in cockroach antennae (Page and Koelling, 2003; Saifullah and Page, 2009) while moth antennae contain their own circadian pacemakers (Schuckel et al., 2007). However, whether another level of modulation in the antennal lobes of mosquitoes may exist in these cases remains unknown.

One key finding of the present study is that for some of the odorants tested, such as benzaldehyde and hexanol, sensitivity was maximal during resting periods. This seemingly paradoxical observation has also been made in other insects (Krishnan et al., 1999; Page and Koelling, 2003), and although various explanations have been proposed, the biological relevance of this phenomenon remains to be understood. Such behavioral rhythms in *Ae. aegypti* may be important for processes such as predator detection or opportunistic feeding or have another role in their complex and nuanced ecology.

In this context, linking these peripheral rhythms in olfactory sensitivity with the behavioral response of the mosquitoes to the same odorants (tested at the same concentrations) appeared necessary. The first conclusion from our behavioral assays is that peaks in olfactory sensitivity do not necessarily align with peaks in behavioral response. For example, *Aedes* mosquitoes were not more repelled at night by benzaldehyde than they were at dusk, even though the electrophysiological responses were higher during the dark phase. Similarly, while EAG recordings showed maximal sensitivity to hexanol at ZT 5–7, no behavioral repulsion was evinced at this time of day. However, in some cases, such as in response to hexanol at night, both behavioral and antennal responses were high. Additionally, both olfactory sensitivity and behavioral repulsion to hexanoic acid were maximal at ZT 10–12. This suggests that the observed modulation of the behavioral output (i.e., the oriented behavior of the insects) is probably resulting from the combined modulation of peripheral and central olfactory processes. This hypothesis is further supported by results obtained in response to octenol, where a clear pattern in behavioral response was observed, in spite of a lack of rhythmicity at the periphery. One factor to be considered in this case is that octenol receptors are located in the maxillary palps of *Ae. aegypti* (Bohbot et al., 2013; Grant and Dickens, 2011). While we found no discernible rhythm in antennal sensitivity, we did observe a significant electrical response to octenol in the EAG recordings that we performed. Therefore, it is possible that *Ae. aegypti* antennal stimulation with octenol at the concentration used (which is high relative to concentrations that mosquitoes would likely encounter in nature) activate nonspecific olfactory receptors (ORs) on the antennae. Interestingly, *Anopheles* mosquitoes also have ORs for octenol located on the maxillary palps (Lu et al., 2007) while also presenting antennal sensitivity to octenol (Cork and Park, 1996; Rinker et al., 2013).

A critical aspect of our experimental design is that it allows us to decouple the rhythm in the behavioral orientation of the insects towards or away from the tested stimuli, from a change of spontaneous locomotor activity levels. Indeed, by taking into account only the mosquitoes that initiate flight and make an active decision between the two test arms of the olfactometer, we ensure that changes in preference indices are not the result of simply having more mosquitoes contributing to the response. In other words, stronger oriented behavioral responses do not mean that more mosquitoes flew, but rather, that among the number of mosquitoes that flew, the responses were more biased towards/away from the odorant stimulus.

Furthermore, an analysis of the proportion of active mosquitoes (meaning mosquitoes making a choice in the olfactometer) revealed that the presence of odorants acted as an activator of behavior. The proportion of active mosquitoes was indeed close or superior to 50%, even at times where spontaneous activity is otherwise usually observed at minimal levels (e.g., ZT 5–7 and 17–19, Figure 2.1 and Figure 2.3). This phenomenon is consistent with what has been previously described (e.g., (Takken et al., 1997)), but we provide here, to our knowledge, the first demonstration of the time-dependent nature of this olfactory activation of behavior. This highlights the importance of the olfactory context when assessing the epidemiological impact of mosquito circadian rhythms.

2.6 Conclusions

In the present study we investigated daily olfactory rhythms in adult female *Ae. aegypti* mosquitoes; specifically, we compared patterns in locomotor activity, olfactory sensitivity, and olfactory behavior. In contrast to previous observations in other mosquito species, we observed an odorant-specific modulation of olfactory sensitivity, decoupled from the rhythms in olfactory behavior. Furthermore, we provide, to our knowledge, the first observation of time-dependence in olfactory activation of behavior in *Ae. aegypti* mosquitoes. Altogether, these results suggest that the olfactory behavior of *Aedes* mosquitoes is modulated throughout the day by both peripheral and central processes. This work sets the basis for future work on mosquitoes that would unravel the mechanisms of central modulation and provide strong impetus for identifying the neural and molecular substrates of the modulation of mosquito olfactory behavior, as they could be leveraged as potential targets to disrupt mosquito-host interactions.

2.7 Author Contributions

Conceptualization, D.F.E. and C.V.; methodology, C.V.; formal analysis, D.F.E. and C.V.; investigation, D.F.E., M.V., E.A.B., K.B., and C.V.; writing—original draft preparation, D.F.E., M.V., E.A.B., K.B., and C.V.; writing—review and editing, D.F.E., M.V., E.A.B., K.B., and C.V.; visualization, D.F.E. and C.V.; supervision, C.V.; project administration, C.V.

2.8 Acknowledgments

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CHAPTER 3: MOLECULAR BASIS OF OLFACTORY RHYTHMS IN *AEDES AEGYPTI*

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(Manuscript in preparation)

3.1 Abstract

Aedes aegypti mosquitoes are the primary vectors of dengue, Zika, and yellow fever viruses. Daily rhythms regulate many of their biological processes and behaviors including their host-seeking. Olfactory processes are primarily responsible for mosquitoes' host-seeking, as they primarily rely on detecting host odor volatiles in the air. We previously observed odor-specific daily rhythms in *Ae. aegypti* female antennal sensitivity and olfactory behavior. For insight into the molecular basis of these observed rhythms, we used RNA-sequencing to carry out a transcriptome wide assessment of gene expression at four times throughout the day in female *Ae. aegypti* heads. RNA-sequencing results indicate several olfactory genes show higher transcript levels during stereotypical locomotor activity troughs (midday and midnight), just ahead of their two activity and host-seeking peaks (dawn and dusk). Since previous studies show that, in *Anopheles* mosquitoes, olfactory protein levels can peak several hours after transcript peaks, transcript levels peaking ahead of activity and host-seeking behavioral peaks in our results suggests that protein levels follow similar trends in *Ae. aegypti* mosquitoes. However, olfactory genes did not all display the same expression patterns, which also aligns with our previous findings that antennal sensitivity rhythms are odor-specific.

3.2 Introduction

Female *Aedes aegypti* respond to human chemosensory cues in order to locate a human host, since they require a blood meal to produce eggs (Lehane, 2005). These cues mediate mosquito-host interactions from a distance, *i.e.*, before mosquitoes land on the host's skin to bite, feed, and potentially transmit pathogens. The mosquito sensory system therefore makes an ideal target for vector-borne disease intervention. As shown in Chapter 2, *Ae. aegypti* females display odor-specific olfactory rhythms. In addition, the mis-aligned rhythms between antennal sensitivity and behavior suggest that there is a secondary level of modulation that takes place in the mosquito brain. However, the underlying molecular mechanisms, which could serve as targets of opportunity to disrupt mosquitoes' attraction to hosts, are yet to be fully characterized. In particular, expression rhythms of genes involved in central and peripheral olfactory processes remain to be distinguished.

Daily rhythms in gene expression, which influence host-seeking behaviors, are a result of circadian and diel regulated gene expression. In other words, rhythmic gene expression is driven by the interaction of internal clocks and the input of cyclic environmental cues. Previous studies using microarray technology have shown that at least 7.9% of the female *Ae. aegypti* head transcriptome is rhythmic under diel conditions including genes involved in a variety of molecular pathways (Leming et al., 2014; Ptitsyn et al., 2011). Similar microarray studies in *Anopheles gambiae* mosquitoes have revealed that rhythms in many olfactory genes can vary greatly based on the light regime (light:dark *vs.* constant darkness) (Rund et al., 2013b). Specifically, many rhythmic genes showed a dramatic decrease in expression under constant darkness or were only rhythmic in diel conditions (Rund et al., 2013b). While both *Ae. aegypti* and *An. gambiae* mosquitoes are prominent disease vectors, they show different behavioral and gene expression patterns, as they occupy different habitats and ecological and temporal niches. For example, *Aedes* are diurnal and thus primarily active during the day, while *Anopheles* are a nocturnal species, with flight, host-seeking, blood-feeding, and reproductive activities all occurring at night. While the aforementioned microarray studies have shed light on rhythmic gene expression in these species of epidemiological importance, it is likely that expression rhythms of genes expressed only in small populations of neurons have been underestimated.

This chapter expands on previous work to provide transcriptome expression levels in the female *Ae. aegypti* head under diel conditions using RNA sequencing, which can achieve a higher resolution of differentially expressed genes and has a lower detection limit as compared to microarray methods (Mantione et al., 2014; Zhao et al., 2014). By analyzing tissue in whole heads, results herein will provide insight into transcript levels throughout the day in both the brain and chemosensory appendages (antenna and maxillary palps).

3.3 Materials and Methods

Mosquito Tissue Sample Collection

Aedes aegypti Liverpool (LVP-IB12, MR4, ATCC®, Manassas, VA, USA) larvae were raised in 26 × 35 × 4 cm covered trays filled with ~1 cm of deionized water, at a density of approximately 200 larvae per tray. They were maintained under light:dark (LD) cycles of 12 h:12 h at 26 °C and 70 ± 10 % humidity. Larvae were maintained on daily feedings of Hikari Tropic First Bites (Petco, San Diego, CA, USA). Pupae were isolated on the day of pupation and placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA). Pupae containers were transferred to opaque plastic containers (Rubbermaid Brute boxes, 71 × 44 × 38 cm) at least three days prior to sample collection to allow mosquitoes to synchronize to a light cycle for sample collection at one of the four tested Zeitgeber time (ZT) periods (0–2, 5–7, 10–12, 18–20 or, in other words, morning, midday, evening and midnight) on a 12 h:12 h LD cycle where lights-on takes place at ZT 0 and lights-off at ZT 12 (Figure 3.1). For each sample, 14–30 adult female mosquitoes aged 4–7 days old were beheaded and whole heads collected and pooled to constitute 1 replicate. Samples at ZT 18–20 were collected under red light illumination, invisible to the mosquito while allowing manipulation by the human experimenter. Each tested ZT included four biological replicates.

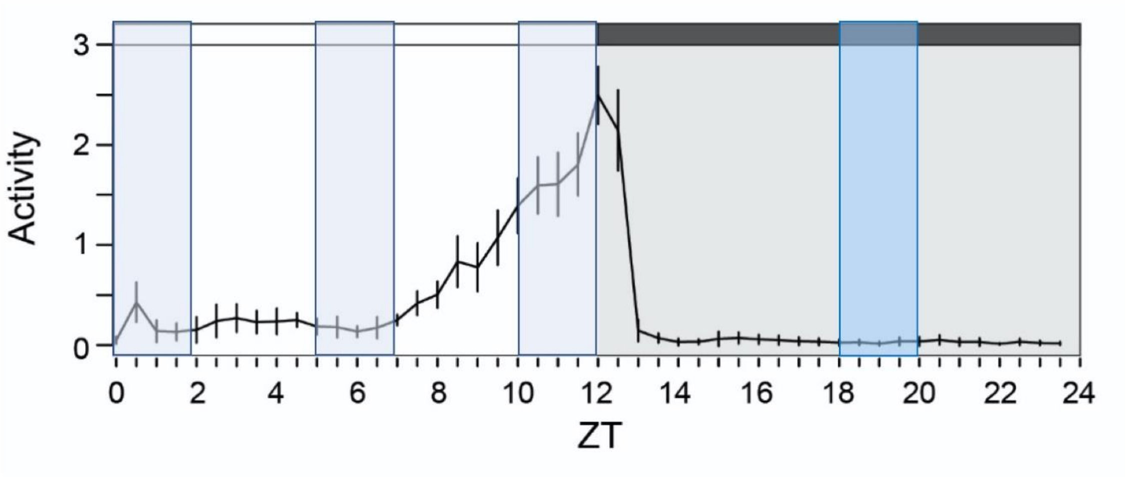

Figure 3.1. Mean locomotor activity pattern of *Ae. aegypti* females in 12 h:12 h light-dark (LD) cycle. Values on the x-axis correspond to Zeitgeber time (ZT). Error bars represent the standard error around the mean. Blue boxes represent the 4 time windows of sample collection. Modified from (Eilerts et al., 2018).

Sequencing

Total RNA was isolated from each sample using the Qiagen (Hilden, Germany) RNeasy Mini Kit following manufacturer's instructions. RNA quality and quantity were evaluated using a Nanodrop (Thermo Scientific, Massachusetts, USA) spectrophotometer and via 1% agarose gel electrophoresis. Samples were sequenced by Novogene (Novogene Corporation Inc., Beijing, China) using Illumina sequencing by synthesis technology. In brief, after quality control assessment of the total RNA, a DNA library is prepared by synthesizing and fragmenting cDNA. Adaptors containing a sequencing binding primer region are attached to each end of the fragments. The libraries are amplified via bridge amplification to produce unique clonal clusters of the template DNA. Complementary DNA is synthesized via DNA polymerase using fluorescent dNTPs, which are detected while synthesized for sequencing. Sequencing results are assessed for quality and mapped back to the reference genome.

Gene Expression Analysis

In order to compare gene expression levels, read counts were normalized by using the Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) or the Transcripts per Million (TPM) units, to account for gene length and sequencing depth. Differential gene expression analysis was performed using the DESeq method, which includes readcount normalization, *p*-value estimation using a negative binomial distribution model, and false discovery rate (FDR) value estimation based on the Benjamini-Hochberg (BH) method (Anders and Huber, 2010; Benjamini and Hochberg, 1995).

Weighted Gene Correlation Network Analysis

In order to compare transcriptomic patterns across all four times-of-day, a weighted gene correlation network analysis (WGCNA) was performed (Langfelder and Horvath, 2008). This method is an unsupervised approach used to identify clusters (modules) of genes whose expression is correlated with experimental treatments (in this case, time-of-day). Gene co-expression networks can reveal relationships between transcripts; for example, modules may reveal biological pathways or link individual gene patterns to global emergent properties of cells, tissue, or organisms functioning as a concerted system (Langfelder and Horvath, 2008). Co-expression modules can provide insights into higher-order transcriptomic organization by creating a biologically meaningful meta-network. Each module, which can contain thousands of genes, is reduced to one representative gene termed an eigengene (Langfelder and Horvath, 2007). RNAseq data was analyzed in R (R Development Core Team, 2020) using Bioconductor software (Huber et al., 2015) and the WGCNA, dynamicTreeCut, and cluster packages (Langfelder and Horvath, 2008; Langfelder and Horvath, 2012; Langfelder et al., 2016; Maechler et al., 2021). Identified modules that showed significant correlation with specific times of day were subsequently used for a gene ontology (GO) analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009a; Huang et al., 2009b).

3.4 Results and Discussion

For all samples, between 91.47 and 92.83 % of total reads were mapped to the reference genome. Uniquely expressed genes at each time of day tested were identified: 45 genes were uniquely expressed at ZT 0–2, 83 at ZT 10–12, 59 at ZT 18–20, and 211 at ZT 5–7 (Figure 3.2a). Non-metric multidimensional scaling (NMDS), shows that biological replicates from the same time of day cluster together (Figure 3.2b). NMDS is an unsupervised non-linear method to extract relationship patterns in gene expression data. This method is preferred over most linear clustering analyses for temporal expression data since NMDS places genes in a continuous space based on rank as opposed to classifying in limited categories (Taguchi and Oono, 2005). In addition to replicates of the same time of day clustering together, the clusters move in a ring-like arrangement around the center in counterclockwise, indicative of temporal expression patterns (Taguchi and Oono, 2005).

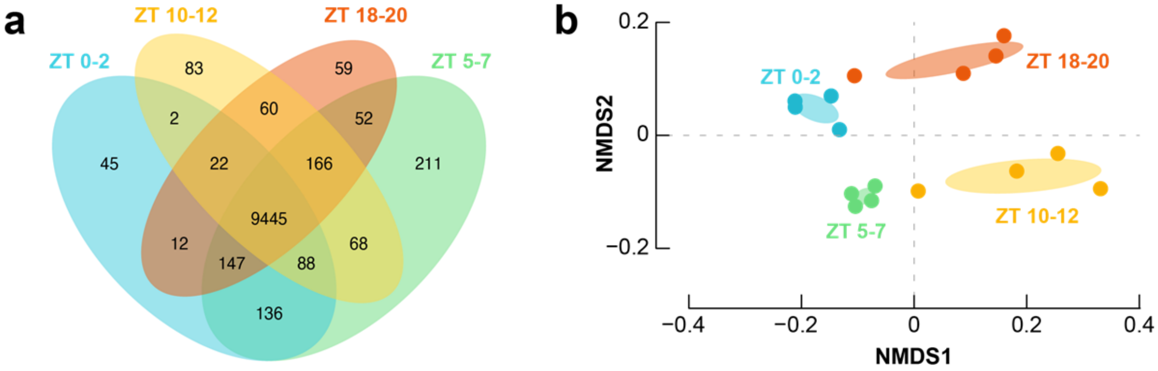

Figure 3.2. Gene expression in *Ae. aegypti* female heads at ZT 0–2, 5–7, 10–12, and 18–20. a) Co-expression Venn diagram showing the number of genes uniquely expressed in each sample group, overlapping regions show the genes that were common between sample groups. b) Non-metric multidimensional scaling (NMDS) of gene expression profiles in female mosquito heads at four times of time (ZT 0–2, 5–7, 10–12, 18–20). Ellipses represent the standard deviation around the centroid of each cluster.

In looking specifically at olfactory genes, we found that global trends for both odorant binding proteins (OBPs) and odorant receptors (ORs) detected tend to show higher transcript levels at ZT 5–7 (Figure 3.3). This was also true for those transcripts that showed differential or unique expression between the times tested (Figure 3.3). Specifically, there were 7 genes encoding OBPs with differential or unique expression and 7 genes encoding ORs with differential or unique expression. Of particular interest is OR4, which shows high transcript levels at ZT 5–7 and 18–20. OR4 is upregulated in human-host preferring mosquitoes and recognizes the human body odor component sulcatone (McBride et al., 2014). Previous work suggests that mosquitoes' evolution of a preference for human body odor in the domesticated form of *Ae. aegypti* mosquitoes (*Ae. aegypti aegypti*) is likely due to increases in expression and sensitivity of OR4 (McBride et al., 2014), which aligns with our findings that OR4 transcript levels are heightened ahead of *Ae. aegypti* peak host-seeking times. Another OR showing differential expression throughout the day in our results is OR10, which shows highest transcript levels at ZT 0–2 and 18–20 (Figure 3.3). OR10 is only expressed in *Ae. aegypti* mosquitoes when they are adults, and is sensitive to indole and narrowly tuned to skatole, a methylated analog of indole (Bohbot and Dickens, 2012; Bohbot et al., 2007). Indole sensitivity within a narrow group of ORs (OR10, OR2, OR9) is the most highly conserved group of ORs across *Ae. aegypti, An. gambiae,* and *Culex quinquefasciatus* mosquitoes, suggesting a critical role in the mosquito life cycle (Bohbot et al., 2011). Indole is a constituent of both animal and plant odors and both indole and skatole have been proposed to mediate oviposition and host-seeking behaviors, but their role in mediating mosquito behavior is likely complex (Blackwell and Johnson, 2000; Cork, 1996; Knudsen et al., 2006; Lee et al., 2015; Meijerink et al., 2000; Ruel et al., 2018). OR10 also shows strong ligand binding and sensitivity to benzaldehyde (Bohbot et al., 2011). While OR10 sensitivity is lower than for indole or skatole, benzaldehyde and 1-octen-3-ol elicited the highest electrophysiological responses in OR10 expressed in *Xenopus* oocytes (Bohbot et al., 2011).

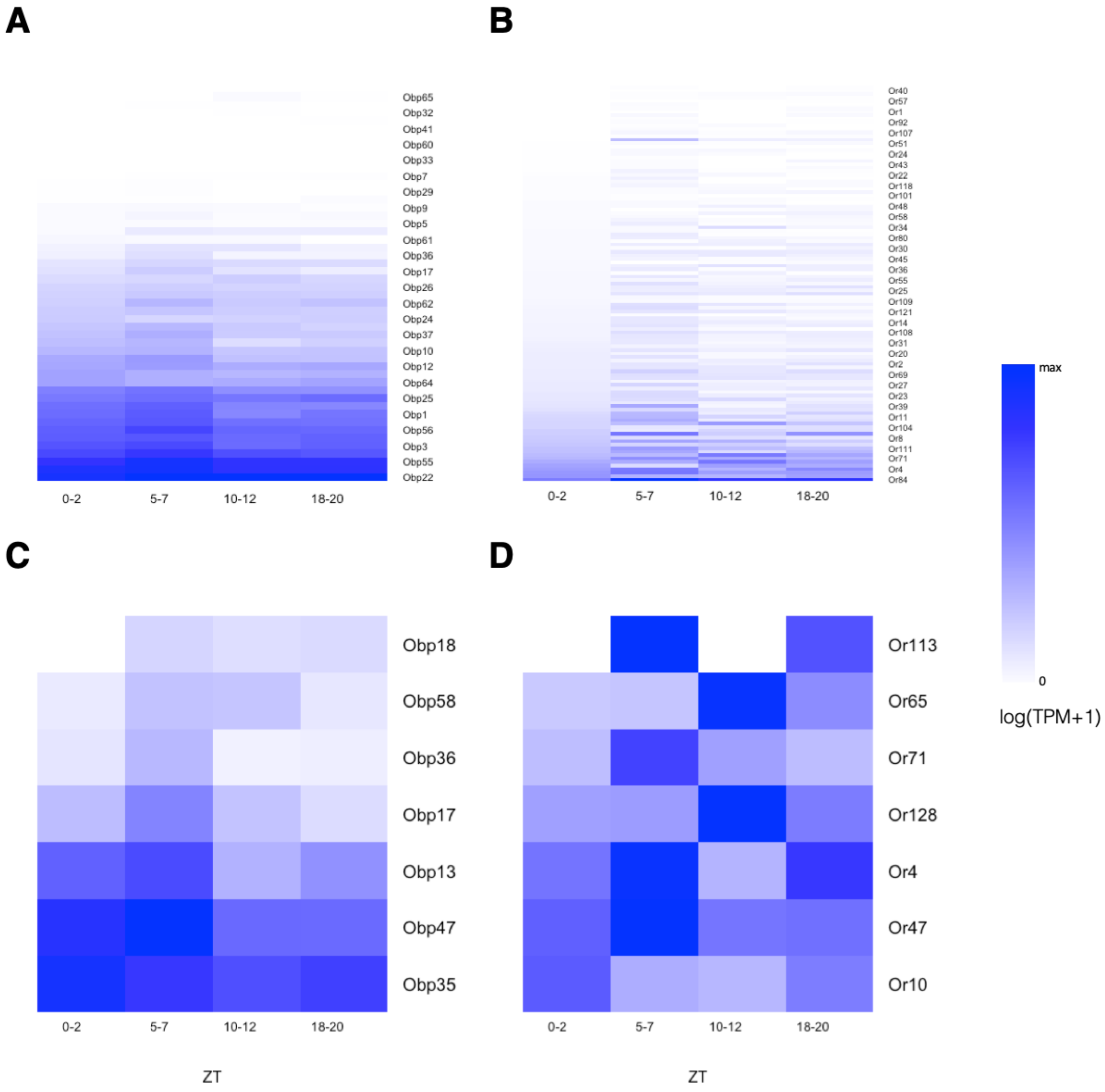

Figure 3.3. Olfactory transcript levels. Expression levels of detected A. OBPs and B. ORs in female *Ae. aegypti* heads at four times of day: ZT 0–2, 5–7, 10–12, 18–20. Expression levels of C. OBPs and D. ORs that were differentially or uniquely expressed between ZT 0–2, 5–7, 10–12, and 18–20.

There are likely some ORs and OBPs that were below the detection limit, as we used whole heads to isolate RNA, and these transcripts would be localized in the antenna and maxillary palps, which would represent a much smaller portion of the total RNA from whole heads. In one recent study using only *Ae. aegypti* antennal tissue for RNAseq analysis, 35 of the 52 annotated OBPs were detected, while 90 of the 97 annotated ORs were detected (Hill et al., 2021). Future studies to characterize transcriptomic rhythms using only antennal tissue would be of interest to get a deeper understanding of rhythms in OBP and ORs not detected in whole head tissue. This presents certain challenges, as antennal appendages are much smaller, more difficult to dissect, and proportionally contain a large amount of cuticle tissue, so sample sizes would need to be quite large to obtain enough tissue for reliable RNAseq results and samples would take much longer to acquire. However, several studies have been successful in performing RNAseq on mosquito antennal tissue (Hill et al., 2021; Maguire et al., 2020; Matthews et al., 2016; McBride et al., 2014; Rinker et al., 2013), so sequencing RNA in *Ae. aegypti* antenna throughout the day to investigate diel and/or circadian rhythms would be feasible, albeit time and labor intensive, and results would certainly be informative for understanding host-seeking rhythms.

The co-expression network based on WGCNA distributed the transcriptomic data into 17 gene modules based on similar expression profiles throughout the day (Figure 3.4). Of the 17 modules, 7 were significantly correlated with time of day (Figure 3.4c). A total of 6 modules were significantly correlated with ZT 5–7 (Fisher's asymptotic p-value for correlation, $p < 0.05$). One module containing 2908 transcripts was significantly correlated with ZT 18–20 (Fisher's asymptotic p-value for correlation, $p < 0.05$). No modules were significantly attributed to ZT 0–2 or ZT 10–12 (Figure 3.4).

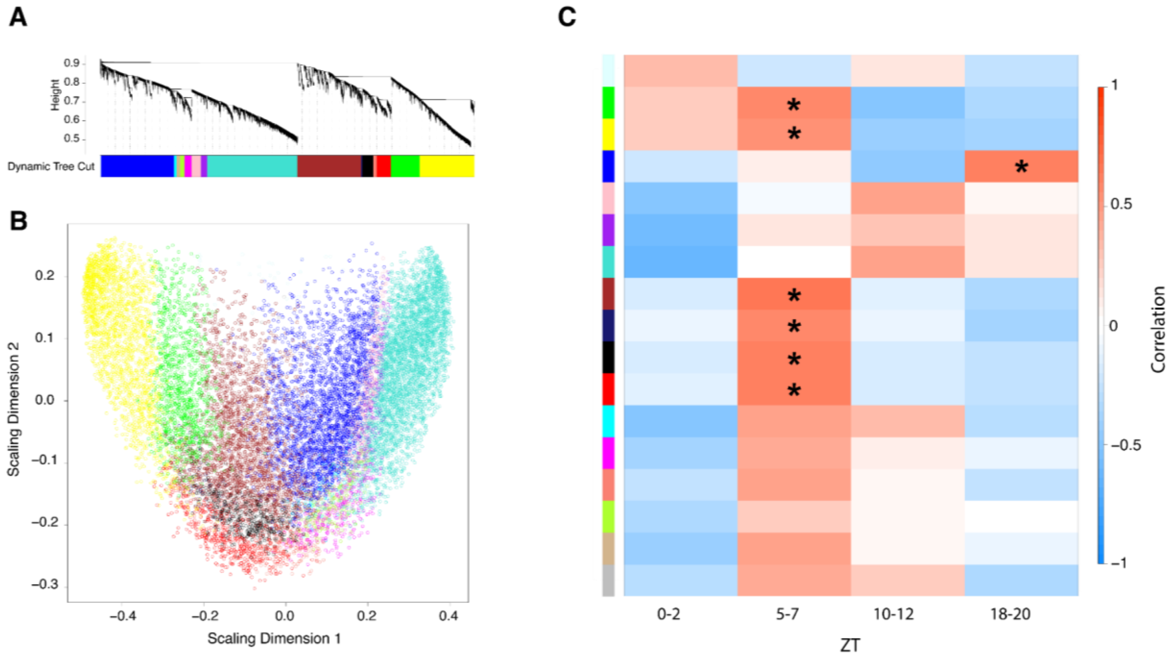

Figure 3.4. WGCNA modules. A. Clustering results of WGCNA modules. B. Multidimensional scaling plot displaying expression data of genes in different modules. C. Heatmap showing correlations between modules and traits.

Many of the modules were highly correlated (Figure 3.4). Many of these modules contain genes pertaining to a specific biological function (Figure 3.5). One gene-specific mechanism that leads to correlated transcription is coregulation either by a common transcription factor (or coregulated transcription factors) (Chapman et al., 2020). By relating modules to gene information and assessing eigengene networks, we can study relationships between pathways.

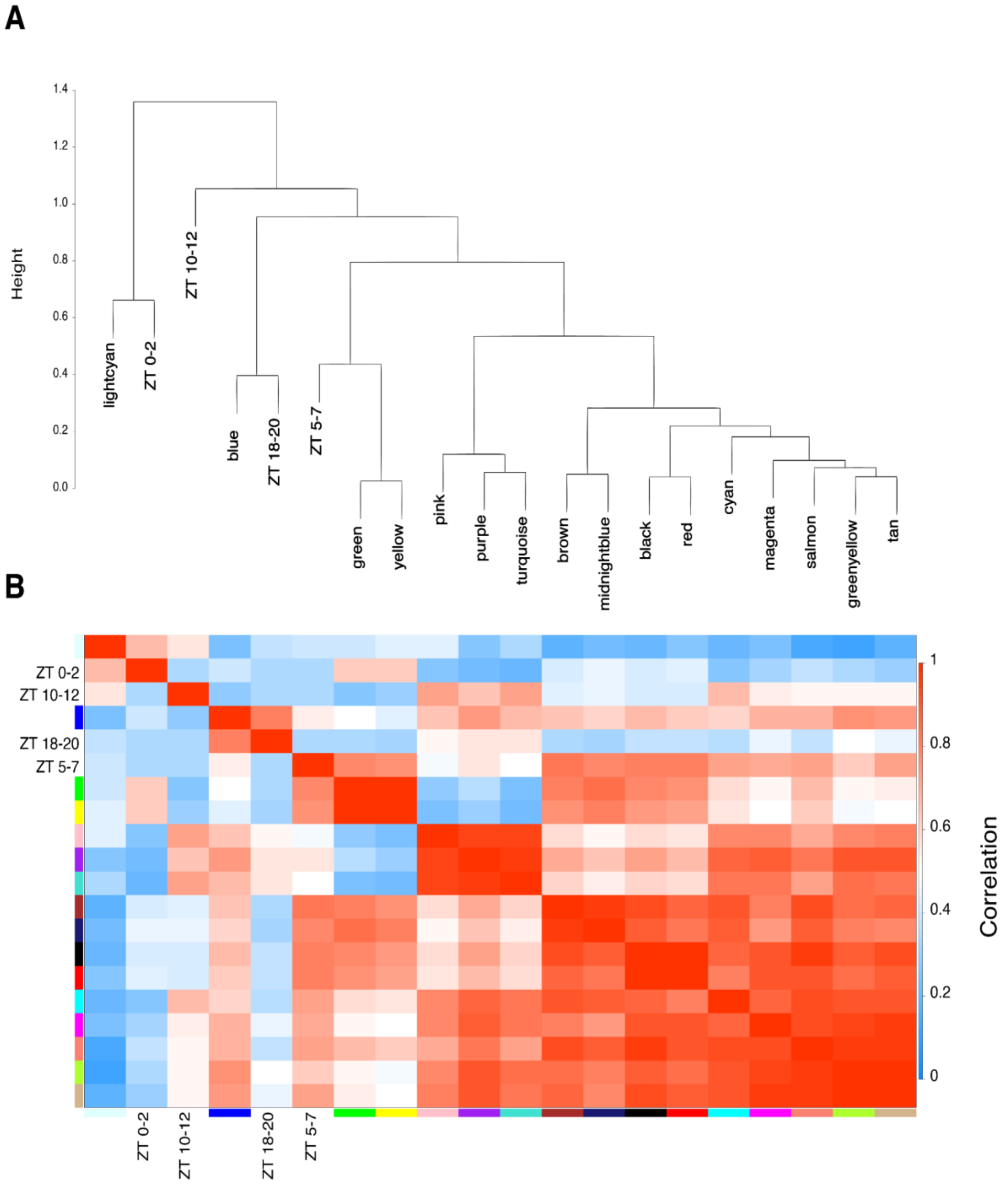

Figure 3.5. Clustering and adjacencies of module eigengenes. A. Unsupervised hierarchical clustering dendrogram of module eigengenes and user-defined time-specific eigengenes. B. Eigengene adjacencies. Each row and column corresponds to one eigengene (labeled by module color, or time-specific user-defined

eigengene). Red indicates high adjacency (positive correlation) and blue indicates low adjacency (negative correlation) as shown in the color legend.

Molecular functions associated with the significant modules notably include many olfactory processes. Specifically, odorant compound and small molecule binding, receptor signaling, transport protein activity, and chemosensory receptor activities (Figure 3.6). Gene ontology analysis of the six significant modules specific to ZT 5–7 contained a significant number of transcripts involved in a variety of biological processes, notably including: translation (green module), signal transduction (GTPase activity, ARF protein regulation, Rho protein regulation, transmembrane receptor protein tyrosine kinase signaling, protein kinase C activity, ATP and GTP binding, ion channels and ion binding; yellow module), olfactory processes (brown module), oxidative stress response (heme and iron binding, monooxygenase activity, oxidoreductase activity, FAD binding; brown and black modules), DNA replication initiation (brown module), chitin catabolism (red module), and carbohydrate metabolism (red module) (Fisher's Exact test (modified as EASE score), $p < 0.05$). Gene ontology analysis of the significant module specific to ZT 18–20 (blue) contained a significant number of transcripts typically involved in chemosensation, as well as protease activity (Fisher's Exact test (modified as EASE score), $p < 0.05$).

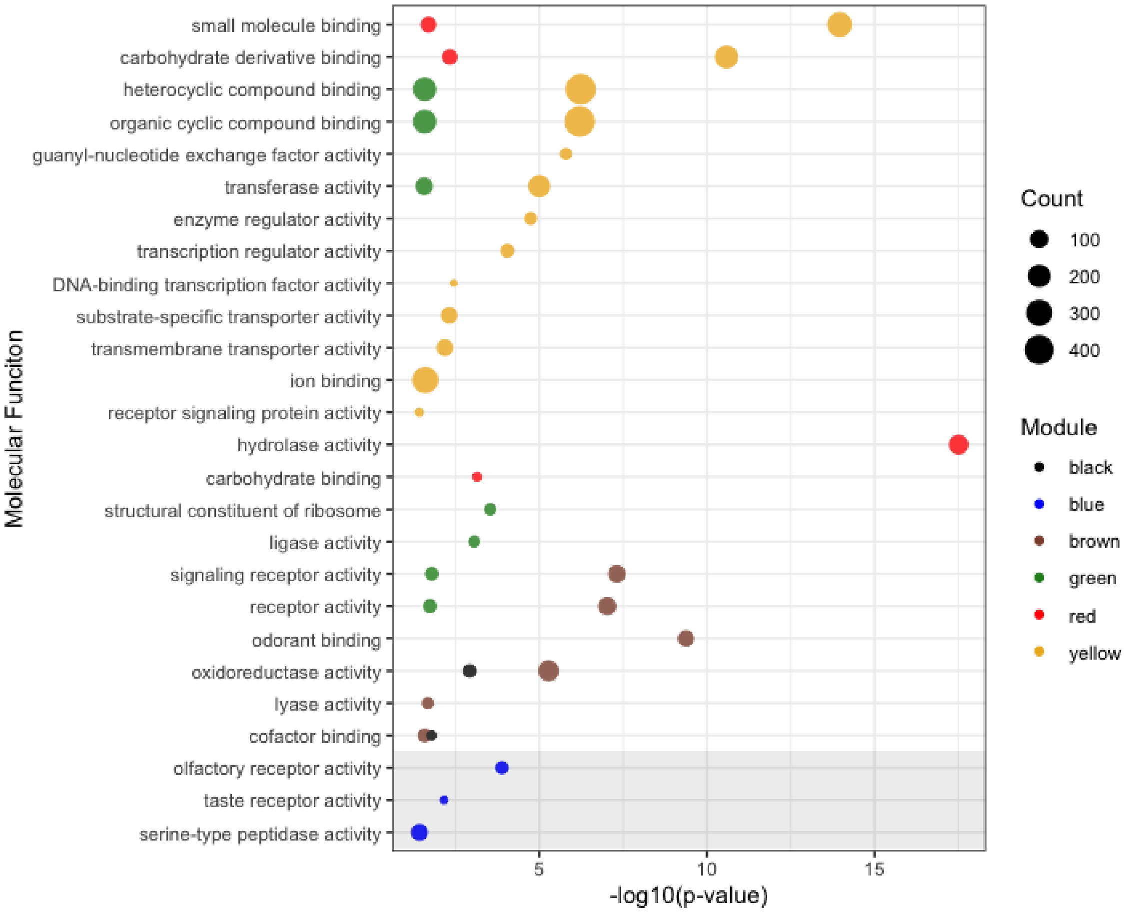

Figure 3.6. Molecular functions associated with modules significantly correlated to ZT 5–7 and ZT 18–20. Count indicates the number of genes per module associated with each molecular function. Modules are notated by color. Modules correlated with ZT 5–7 are shown with a white background, and the module correlated with ZT 18–20 is shown with a gray background.

Higher expression levels of translation proteins in the middle of the day could be beneficial as the mosquito is preparing for amplified levels of protein synthesis during the end of the day and/or into the night, such as those necessary to efficient host seeking, flight, rebuilding of cellular components during night time resting, or potentially investing in the synthesis of proteins involved in blood digestion and metabolism. Previous literature has shown that, in *An. gambiae,* there is a significant lag between OBP RNA rhythms and OBP protein rhythms (almost 7 hours), so it's worth noting that transcriptomic patterns we observed might not be representative of protein levels at that time (Rund et al., 2013a). It's likely that some transcripts we observed in *Ae. aegypti* heads have similar delays in peak protein expression, and this would align with *Ae. aegypti* host-seeking locomotor and flight activity, which peaks in the hours following ZT 5–7 until the end of the subjective day or light regimen (ZT 12). It's also important to note that many *Aedes* ORs are not de-orphanized, so it's unclear what odor ligands they bind to, and ORs do play a role outside of host-seeking, such as for sugar-feeding or oviposition.

3.5 Conclusions

RNA-sequencing results indicate that many olfactory genes (OBPs and ORs) show higher transcript levels during stereotypical locomotor activity troughs (ZT 5–7 and 18–20, or midday and midnight). This likely represents that key olfactory genes that mosquitoes rely on for successful host-seeking are expressed during activity peak times (dawn, dusk), since protein levels likely lag behind transcript levels, as is the case for *An. gambiae* OBPs (Rund et al., 2013a). Future research to quantify *Ae. aegypti* OBP and OR protein levels throughout the day, and compare to transcriptomic rhythms, would therefore be informative. Additionally, further research focusing specifically on the brain, antennae, and maxillary palps would allow us to differentiate between neural processes and odorant transport and binding in the olfactory appendages. Since the antenna and palps are much smaller organs as compared to the rest of the mosquito head, some transcripts in these tissues may not be detected by quantifying transcripts in whole heads. While most *Ae. aegypti* ORs have not been de-orphanized and their ligand specificity remains unknown, results from Chapter 2 showed that antennal sensitivity to specific odors peak at different times of day, so anntenal specific transcriptome analysis could provide insight into the genes critical for odorant-specific differences in antennal sensitivity.

3.6 Author Contributions

Conceptualization, D.F.E. and C.V.; methodology, D.F.E, J.B., and C.V.; formal analysis, D.F.E. and C.V.; investigation, D.F.E., J.B., and C.V.; writing—original draft preparation, D.F.E.; writing—review and editing, D.F.E. and C.V.; visualization, D.F.E. and C.V.; supervision, C.V.; project administration, C.V.

CHAPTER 4: PLASTICITY OF *AEDES AEGYPTI* HOST-SEEKING BEHAVIORS

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(Manuscript in preparation)

4.1 Abstract

While *Aedes aegypti* mosquitoes are typically active and host-seeking at the start and end of the day, they can also host-seek and bite outside of their stereotypical activity peaks. We previously observed that female *Ae. aegypti* exhibit an odor-induced activation of behavior and showed higher activity levels at the start of the day when presented with an odor, as opposed to their stereotypical largest peak in activity being at the end of the day. To investigate the plasticity of female *Ae. aegypti* olfactory rhythms, and determine if they are capable of synchronizing their activity rhythms to olfactory cues, we developed a custom 3D-printed odor manifold to expose mosquitoes to a host odor (octenol) in the morning while monitoring their individual activity levels throughout the day. We found that previous exposure to host odor cues outside of stereotypical peak activity times caused activity levels throughout the day to increase in female *Ae. aegypti* mosquitoes. The implication that mosquitoes exposed to host cues will alter or increase activity suggests that stereotypical activity patterns obtained in laboratory experiments may not be representative of domesticated mosquitoes' activity, and their behavioral plasticity should be accounted for in determining vectorial capacity.

4.2 Introduction

Aedes aegypti mosquitoes are diurnal and are typically most active at the start and end of the day, when they are more likely to be host-seeking and biting (Eilerts et al., 2018; Jones, 1981; Kawada et al., 2005; Lima-Camara, 2010; Taylor and Jones, 1969). However, *Ae. aegypti* are an anthropophilic domestic species often exposed to artificial light and can host seek or bite humans outside of their stereotypical activity peak times (Day, 2005; Rund et al., 2020). Female mosquitoes rely primarily on their sense of olfaction to identify a host, but must be particularly responsive to host cues in circumstances when they are most likely to be successful in obtaining a blood meal, benefit from it, and avoid predation (Gadenne et al., 2016; Wynne et al., 2020). Their behavioral response to odor cues is modulated by expression of chemosensory genes encoding odorant binding proteins such as (OBPs), odorant receptors (ORs), and ionotropic receptors (IRs) (Tallon et al., 2019). Since ORs bind discriminately to specific compounds, expression levels of such key olfactory proteins leads to variations in antennal sensitivity to specific odors (Rund et al., 2013a).

As shown in Chapter 2, *Ae. aegypti* females exhibit an odor-induced activation of behavior. Specifically, they were more active (more likely to make a choice in the Y-maze experiments) when presented with an odor at the beginning of the day, despite their stereotypical spontaneous main activity peak being at the end of the day. In addition, in Chapter 3, we saw that the expression of several chemosensory genes varied throughout the day. However, whether mosquitoes can synchronize their activity pattern to periodic olfactory stimulation remains unknown. Therefore, the objective of this chapter was to determine the plasticity of mosquitoes' olfactory rhythms. Since spontaneous *Ae. aegypti* activity typically exhibits a morning peak and an end of the day peak but they primarily host-seek at the end of the day, the studies in this chapter are aimed at determining the mosquitoes' propensity to host-seek during morning and midday times and adjust their behavior in response to the cyclic exposure to host cues.

4.3 Materials and Methods

Mosquito Rearing

Female *Ae. aegypti* Rockefeller (MR-734, MR4, ATCC®, Manassas, VA, USA) were used for all experiments. Larvae were raised in a 26 × 35 × 4 cm covered tray filled with ~1 cm of deionized water, at a density of approximately 200 larvae per tray. Mosquitoes were maintained under light:dark (LD) cycles of 12 h:12 h at 26 °C and 70 ± 10% humidity. Larvae were fed a daily diet of Hikari Tropic First Bites (Petco, San Diego, CA, USA). Pupae were isolated on the day of pupation and placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA).

Y-maze two-choice assay for olfactory behavior

Isolated pupae in breeding containers were transferred to opaque plastic containers (Rubbermaid Brute boxes, 71 × 44 × 38 cm) at least three days prior to experiments to allow mosquitoes to synchronize to a light cycle aligned with the experimenters' work hours for one of the two tested Zeitgeber time (ZT) periods on a 12 h:12 h LD cycle where lights-on takes place at ZT 0 and lights-off at ZT 12. Six to 8 day old female mosquitoes were used for olfactory experiments. An enclosed Y-maze olfactometer was used as previously described (Eilerts et al., 2018) to quantify the behavioral responses of mosquitoes to a host odor blend and plant odor blend during the first two hours and the last two hours of the photophase (ZT 0–2 and ZT 10–12). Briefly, the olfactometer consisted of clear acrylic with two choice arms and an arm connected to a central chamber with a constant filtered laminar airflow originating at the distal end of each choice arm. Olfactory stimuli were delivered at the same end of each choice arm by separate filtered airflows that passed through vials containing either the test odor or vehicle. All experiments were performed at a constant temperature (26 ± 2 °C) and relative humidity (50–70%). The human odor blend consisted of 3.5 mM lactic acid, 3.7 mM nonanal, 1.18 mM 1-octen-3-ol, and 0.34 mM butyric acid in mineral oil. The plant odor consisted of 0.6793 mM octanal, 0.541 mM benzaldehyde, 5.0431 mM linalool, and 0.5976 mM beta-pinene in mineral oil. For each odor and time point, 38 mosquitoes made a choice and were considered for preference analysis.

Feeding behaviors and locomotor activity after repeated exposure to host cues

Four-day-old male and female mosquitoes (approximately 150–250 total per group; 4 groups total) in a 12 x 12 x 12 inch collapsible insect mesh cage were starved for 2 hours, then allowed to membrane feed on 10% sucrose solution warmed to 37 °C for 30 min at ZT 5. During this time, a filter paper laden with approximately 1 mL of a host odor blend (lactic acid, nonanal, 1-octen-3-ol, and buyric acid in mineral oil, at ecologically relevant concentrations of 3.5 mM, 3.7 mM, 1.18 mM, and 0.34 mM, respectively) was placed on top of the cage, at proximity to a glass artificial feeder (Lillie Glassblowers, Smyrna, GA). Mosquito behavior at the membrane feeder was recorded with a video camera (Model C920, Logitech, Newark, CA, USA) for the duration of the 30 min period. This was repeated daily for a total of 5 days, at the same time each day. After recording was completed on the 5th day, locomotor and flight activity of female mosquitoes from the test group were recorded using a locomotor activity monitor (Trikinetics LAM25, Waltham, MA, USA) as previously described (Eilerts et al., 2018). Briefly, individual mosquitoes were placed in glass cylindrical tubes with access to 10% sucrose at one end and tubes were positioned through the openings on the panel so that the infrared beams were located at the approximate center of each tube. Daily locomotion was recorded as the number of beam crossing per 10 min interval. All recordings occurred in a light-proof enclosure with its own lighting system, which consisted of a light-emitting diode (LED) light (800 Lumen, Philips, Amsterdam, The Netherlands) timed to a 12 h:12 h LD cycle. Data analyses were performed on locomotor activity over 12 consecutive days following the 5 days of host cue exposure and sugar-feeding. Individuals that were found dead at the end of the experiments were discarded from calculations after the last timepoint being recorded as active.

Host odor entrainment with the odor manifold

Individual female mosquitoes were placed in glass cylindrical tubes (25 mm diameter x 125 mm length, Trikinetics, Waltham, MA, USA) at 3-5 days old. One end of the tubes was fitted with a custom 3D-printed cap made of clear resin (Formlabs Inc., Somerville, MA) with a hole to accommodate a reservoir containing a cotton plug with 10% sucrose, as well as surrounding air holes for ventilation (Figure 4.1). The other end of the tube was fitted in a custom 3D-printed odor manifold which allows airflow and gas delivery through the tubes. A constant airflow was established via odor-resistant polyvinyl chloride (PVC) tubing (McMaster-Carr, Elmhurst, IL, USA) leading to the manifold and glass tubes via a single inlet on the manifold. The tubing passed through a scintillation vial containing the tested odor. Locomotor and flight activity of female mosquitoes were recorded using a locomotor activity monitor (Trikinetics LAM25, Waltham, MA, USA), in which the tubes were threaded through holes in the LAM25 circuit board, where 3 infrared beams and detectors bisect each tube. The odor manifold and activity monitor were contained in a light-proof enclosure with an air outlet funnel and its own lighting system, which consisted of a light-emitting diode (LED) light (800 Lumen, Philips, Amsterdam, The Netherlands) timed to a 12 h:12 h LD cycle aligned to the mosquitoes' rearing conditions. Mosquitoes were maintained in the tubes for a duration of 8 days following the date of experimental setup, with their activity recorded for the duration of the 8 days. Mosquitoes were exposed to a 30 minute odor pulse starting at ZT 1 on days 3–6, and exposed to a solvent-laden (*i.e.*, mineral oil) air flow on days 1–2 and 7–8. Daily locomotion was recorded as the number of beam crossing per 10 min intervals using the DAMSystem3 Software (Trikinetics, Waltham, MA, USA). Individuals that were found dead at the end of the experiments were discarded from calculations after the last timepoint being recorded as active, and removed from pairwise comparisons.

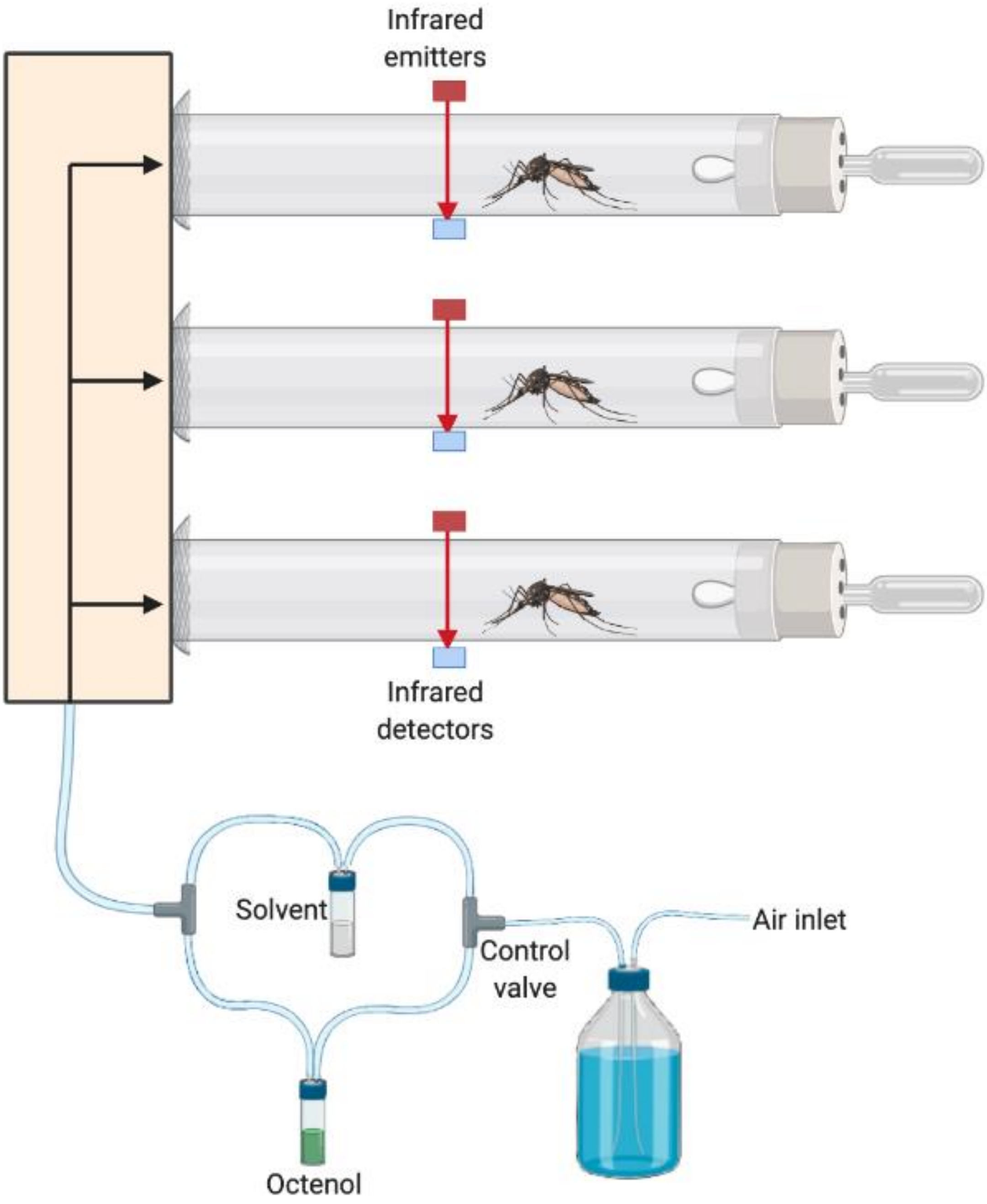

Figure 4.1. Schematic representation of the odor manifold and activity monitoring system. A humidified airflow was established and lead to a control valve that directs the air to either a scintillation vial containing the solvent (mineral oil) or octenol, before being directed to the manifold inlet, which then directs air to each tube threaded through a locomotor activity monitor (Trikinetics LAM25,

Waltham, MA, USA). The monitor contains 3 infrared beams and detectors that bisect each tube. One end of the tubes is enclosed with a piece of mesh and inserted into an air outlet in the manifold, and the other end was fitted with a custom cap with one central hole to accomodate a 10% sucrose reservoir and surrounding smaller air holes for ventilation.

Data Analysis

Binary data obtained in the olfactometer were compared to chance by means of the Binomial Exact test (α = 0.05). CowLog software was used to code behaviors from the video recordings for later analysis (Pastell, 2016). This coded and time-stamped behavioral data, as well as locomotor activity data, and binary data collected in the olfactometer were analyzed in the R programming language (version 4.0.3 (R Development Core Team, 2020)). Landing, probing, and feeding behaviors were identified visually; probing was identified via head movements and feeding was identified when a mosquito remained unmoving with their proboscis in the feeding membrane for five or more seconds after probing. Behavioral data was processed by visually identifying each behavior (landing, probind, feeding) recorded and summing the instances over a 30 minute period. The frequency of behavioral events was normalized to the maximum number of behaviors observed over the five days for each replicate. Activity data was analyzed by calculating the William's mean (Mw) (Haddow, 1960). Activity data for individuals observed dead at the end of the experiment were excluded.

4.4 Results and Discussion

Ae. aegypti females are typically active in the early morning and late evening. In order to determine what odorant blend to use for our odor entrainment experiments, we first used a Y-maze olfactometer to examine behavioral preferences between host and plant odors during their two stereotypical activity peaks (ZT 0–2 and 10–12, dawn and dusk, respectively) (Figure 4.2). At both times of day, mosquitoes showed a moderate preference for the human odor blend, represented as positive preference index values, although the responses were not significantly different from chance (Figure 4.1B; n=38, 61% attraction; Binomial Exact test, p = .128). Activity levels, or decision-making, in the olfactometer (i.e., the proportions of mosquitoes initiating flight and making a choice between one of the two decision arms), were consistent across the two tested times of day (Figure 4.2C). These results indicate that despite the typically more prominent peak in locomotor activity at the end of the day, they exhibit similar behavioral responses to host and plant odors. Therefore, mosquitoes may be just as likely to host or sugar-seek at both times in the wild. However, given the tendency of mosquitoes to prefer the host blend, we opted for host odors to test their ability to entrain to olfactory cues.

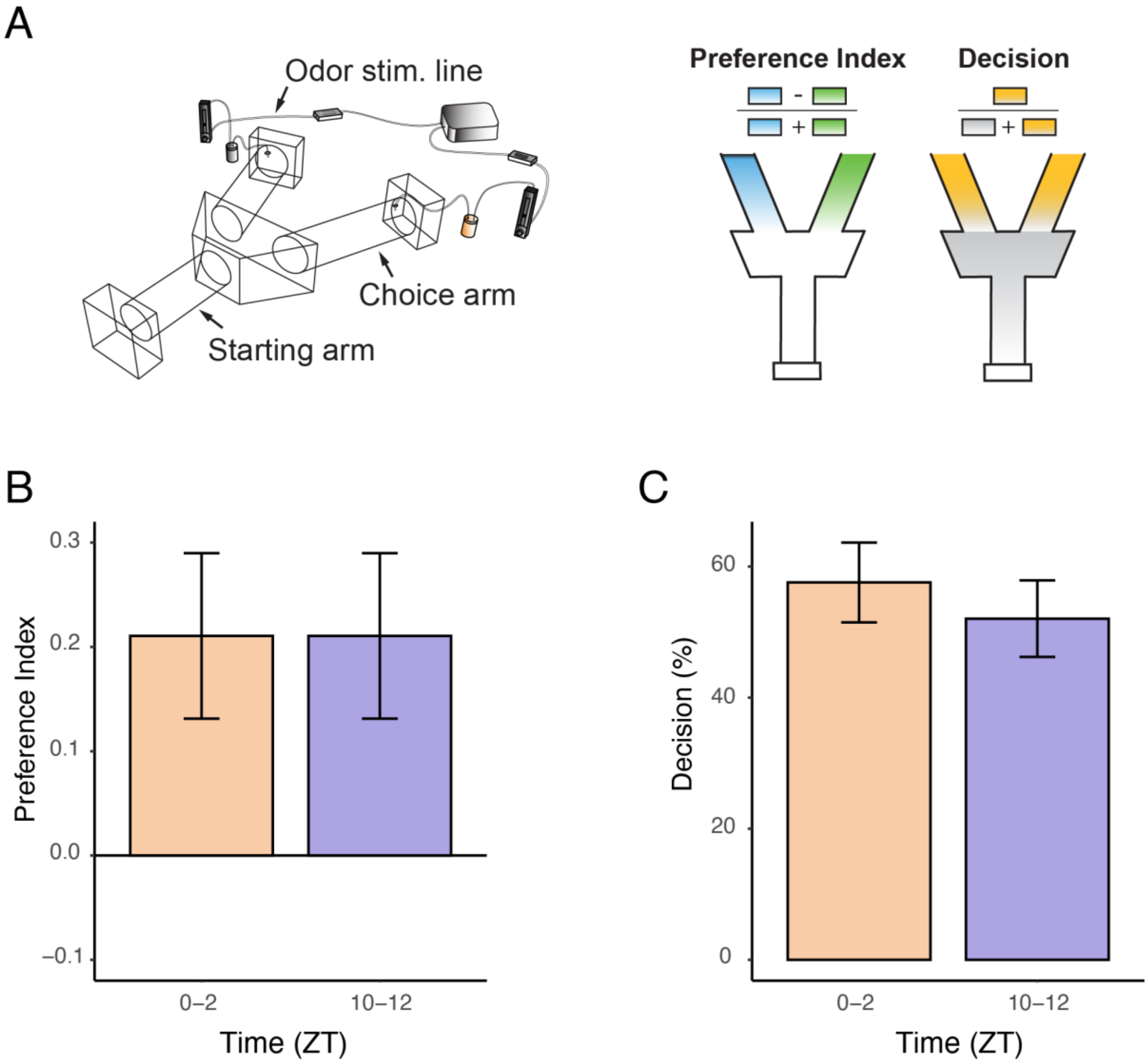

Figure 4.2. Behavioral response to host and plant odors at ZT 0–2 and 10–12. A. Schematic representation of the Y-maze two-choice olfactometer. Mosquitoes are released into the entrance arm and fly upwind where they are forced to make a decision between the two choice arms, one delivering the host odor stimulus, and one delivering the plant odor stimulus. B. Mosquito odor preference represented as a preference index. Behavioral responses are positive, indicating more mosquitoes chose the human odor arm, but results were not significantly different from chance (Binomial Exact test, p = 0.128). C. Percentage of mosquitoes that made an active choice between the two choice arms of the olfactometer at two

times of day (ZT 0–2, 10–12). Decision-making, or activity, was not significantly different between these two times (p > .1, pairwise proportion test). Error bars represent the standard error to the mean.

During the five-day period mosquitoes were exposed to host cues in the behavioral exposure assay, the frequency of mosquito probing and feeding behavior increased after the second day of treatment, but was not statistically significant (Tukey post-hoc test, p > 0.1; Figure 4.3B). Locomotor behavior of individualized female mosquitoes after this treatment period also showed similar daily activity patterns as compared to age-matched females who were not exposed to host cues (Wilcoxon test, p > 0.1; Figure 4.3A,C). While there was no obvious change to female mosquitoes' locomotor and flight activity as measured by the activity monitors, this is likely because this experiment design resulted in a change of context from a cage with hundreds of male and female mosquitoes to much smaller tubes of individualized female mosquitoes.

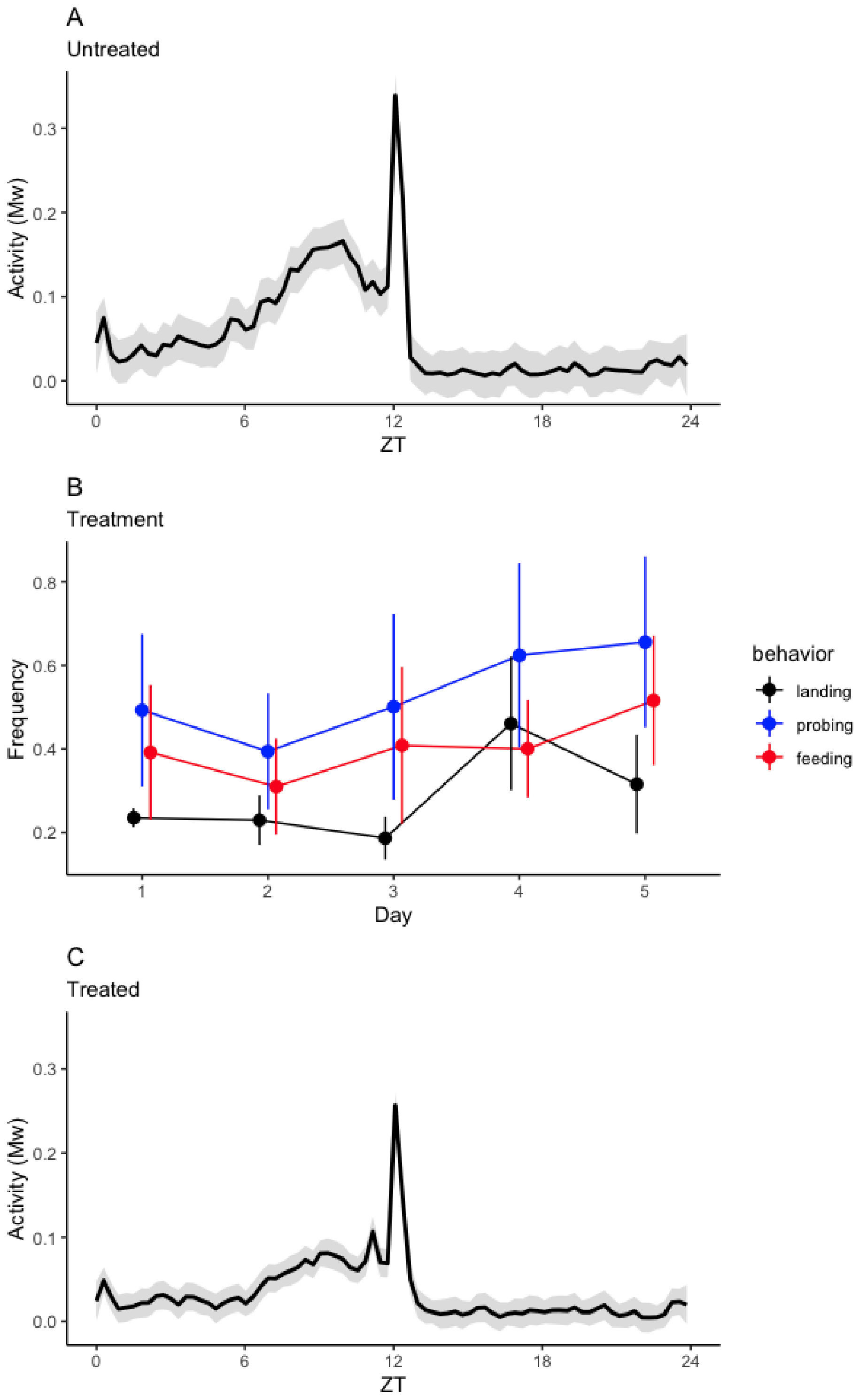

Figure 4.3. Activity and behavioral patterns of mosquitoes exposed to host cues. A. Average daily locomotor and flight activity patterns of untreated

8-day-old *Ae. aegypti* females for 7 days. B. Frequency of landing, probing, and feeding behaviors of mosquitoes exposed to a host odor blend and warmed sucrose feeder for 30 min at ZT 5 for five consecutive days. Dots indicate the mean frequency per day and bars represent the standard error of the mean. C. Average daily activity and locomotor patterns of mosquitoes exposed to host odor cues in (B) for 7 days. Black lines in A and C indicate the average activity, calculated by determining the William's mean (Mw) and gray shading indicates the standard error of the mean.

In order to control for changes of experimental context, we developed a custom 3D-printed odor manifold, so that host odor exposure occurred directly in the activity monitor tubes. There was thus no change in experimental context and handling of the mosquitoes between exposure and activity monitoring was prevented. This also allowed for higher resolution activity monitoring before and during treatment time. For experiments with the odor manifold, we used 65 mM octenol since this host related odor has been shown to be learnable by *Ae. aegypti* and our previous results show that they are attracted to octenol at this concentration (Chapter 2) (Eilerts et al., 2018; Vinauger et al., 2014). Mosquitoes were exposed to a solvent-laden (mineral oil) air flow throughout the experiment; for four days starting on the third day, they were exposed to a 30-minute pulse of octenol starting at ZT 1 (Figure 4.4). On the days with the odor pulse and days following the pulse days, we observed an increase in mosquitoes' general activity patterns during the day (Figure 4.4, 4.5A).

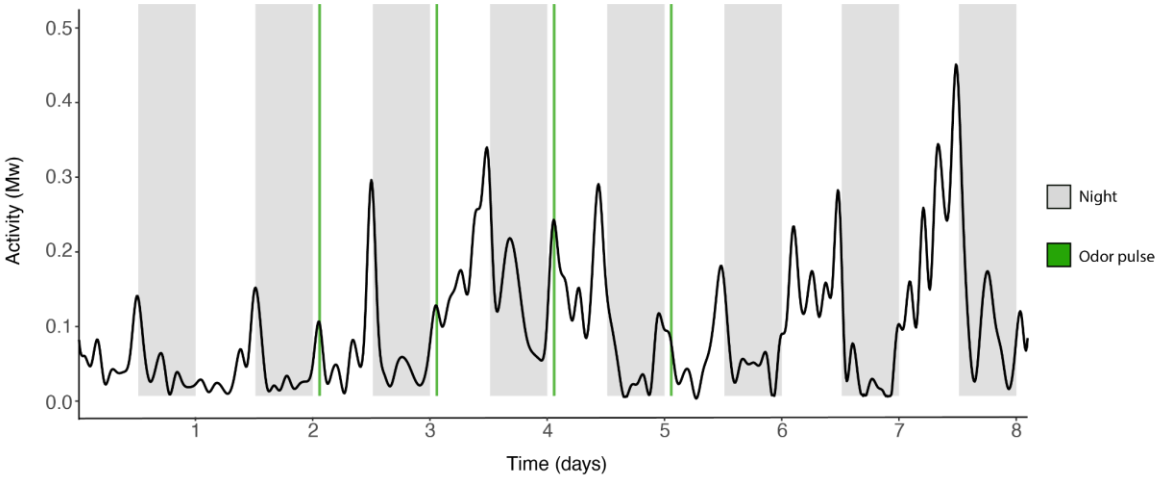

Figure 4.4. Locomotor activity of *Ae. aegypti* females exposed to 8 days of octenol odor pulses. Activity was recorded under LD 12:12 (gray indicates dark periods) and Days 3-6 included a 30-min odor pulse of octenol at ZT 1 (green) (n = 24). The black line represents locally estimated scatterplot smoothing (LOESS) of the William's mean (Mw) of the activity of all individuals.

We quantified the mean individual activity of mosquitoes before, during, and after the days they were exposed to the octenol odor pulse at six different times of day: the period of daytime before the odor pulse was initiated (ZT 0–1), the midday time period corresponding to stereotypical *Ae. aegypti*'s daytime activity trough (ZT 1–6), the end of day time period corresponding to *Ae. aegypti*'s activity peak (ZT 6–11), the transition from day to night, which typically causes a spike in activity following lights-off in laboratory experiments (ZT 11–13), the two nighttime periods (ZT 13–18 and 13–24) following lights-off and before lights-on, respectively (Figure 4.5).

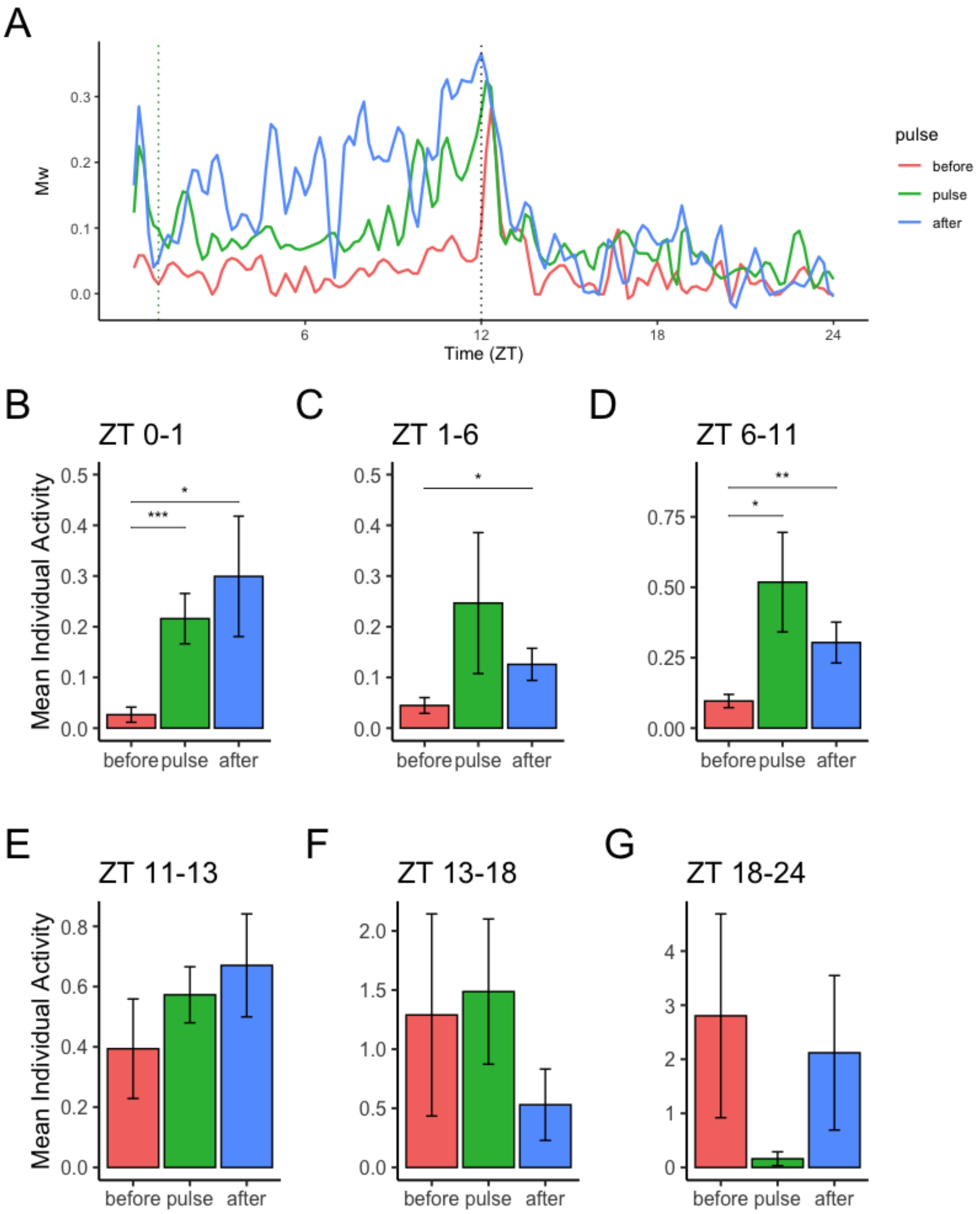

Figure 4.5. Daily and individual locomotor activity of *Ae. aegypti* females in the odor manifold. A) Mean daily activity of all mosquitoes before (pink), during (green), and after (blue) exposure to a 30 min odor pulse of octenol at ZT 1 (first dotted line). B) Mean individual activity of mosquitoes before (pink), during

(green), and after (blue) exposure to the odor pulse at ZT 0–1, C) ZT 1–6, D) ZT 6–11, E) ZT 11–13, F) ZT 13–18, and G) ZT 13–24. Error bars represent the standard error of the mean and pairwise comparisons were performed using a *t*-test. Asterisks represent significance level (* : $p < .05$; ** : $p < .01$; *** : $p < .001$) .

We observed an increase in mean individual activity during and after pulse days during all daytime periods, with significant increases in activity in the days following the pulse at ZT 0–1, 1–6, and 6–11 (Figure 4.5B-D, $p < .05$, *t*-test). During the stereotypical activity peak times in the absence of any odor (ZT 0–1 and 6–11), there was also a significant increase in mean individual activity during the pulse days (Figure 4.5B,D, $p < .05$, *t*-test). These results suggest that mosquito activity is plastic and entrainable to host odor cues. Human behavior indeed influences domestic mosquito behaviors, as has been shown by the influence of artificial light at night (Rund et al., 2020) and behavioral shifts in biting time or site following insecticide use (Carrasco et al., 2019). Spontaneous locomotor activity is often used as a proxy for mosquito host-seeking and biting frequency, but spontaneous activity does not account for behavioral plasticity in response to human behaviors or host cues in an urban context. Specifically, while the end of the daytime period is considered to be the peak host-seeking and biting time for *Ae. aegypti* females, experiments in this Chapter in combination with results from Chapter 2 suggest that the early morning hours may represent an equal or greater risk of biting frequency for domesticated mosquitoes that may detect host cues at different times of day.

4.5 Conclusions

In both this Chapter as well as Chapter 2, we observed an activation of mosquito locomotor behavior. We found that previous exposure to host odor cues, even outside of stereotypical peak activity times at the end of the day, causes an increase in female *Ae. aegypti* activity throughout the day. Accounting for such plasticity in olfactory behavior is important in order to adequately address the epidemiological consequences of urbanization and human behavior.

4.6 Author Contributions

Conceptualization, D.F.E. and C.V.; methodology, D.F.E, K.C., and C.V.; formal analysis, D.F.E. and C.V.; investigation, D.F.E., K.C., O.E., and C.V.; writing—original draft preparation, D.F.E.; writing—review and editing, D.F.E. and C.V.; visualization, D.F.E. and C.V.; supervision, C.V.; project administration, C.V.

CHAPTER 5: DIET-INDUCED CHANGES TO *AEDES AEGYPTI* ACTIVITY PATTERN

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(Manuscript in preparation)

5.1 Abstract

Female mosquitoes require a blood meal to obtain nutrients and proteins for egg development. Blood-feeding, however, can expose mosquitoes to infective pathogens, as well as a variety of stressors such as thermal stress, osmotic stress, or oxidative stress when heme-containing proteins are digested. Excess free heme and iron can lead to oxidative stress by promoting the productive of reactive oxygen species, which can be toxic and cause cell damage or death. In the model organism *Drosophila melanogaster*, oxidative stress management genes are controlled by the circadian clock. Additionally, the clock transcription factors that maintain daily circadian rhythms are also redox-sensitive in *D. melanogaster*. It's unclear if this bidirectional relationship between oxidative stress management and the circadian clock functions similarly in mosquitoes, but it's been shown that blood-feeding disrupts normal activity patterns. To investigate the role of diet heme on observed *Ae. aegypt*i female mosquito activity after feeding, we compared activity patterns of blood and sugar-fed mosquitoes to that of mosquitoes fed on two artificial blood protein mimic diets: one containing hemoglobin and one without. We additionally investigated expression of key genes involved in modulating host-seeking behaviors, to determine if feeding on a heme-containing diet impacts transcript abundance. We found that diet hemoglobin does indeed play a role in activity reduction after feeding, as well as transcription of several genes. Hemoglobin caused decreased transcription of the circadian pacemaker gene *period* in female *Ae. aegypti* heads, but the impact on peripheral clock genes remains ambiguous. We also further show that *Ae. aegypti* exhibit mechanisms of oxidative stress management unique from non-blood-feeding insects. Overall, our results indicate that there is a complex network of interactions between multiple genes or pathways

that play a role in modulating behaviors after feeding in *Ae. aegypti* females. Since blood-feeding is critical to mosquitoes' reproduction and role as vectors, understanding the unique and complex mechanisms that allow mosquitoes to cope with blood meal stressors is of great importance and may present novel targets for vector control.

5.2 Introduction

Both male and female adult mosquitoes can survive by feeding on carbohydrates (sugars) that they acquire by ingesting plant nectar, juices from ripe fruits, and honeydew (Barredo and DeGennaro, 2020; Foster, 1995). These carbohydrates are metabolized to provide energy for flight, host-seeking, and reproduction (Barredo and DeGennaro, 2020). However, adult female mosquitoes require a blood meal to obtain the necessary proteins and nutrients for egg development (Greenberg, 1951). When blood-feeding, mosquitoes may be exposed to blood-borne pathogens in the blood meal. If a mosquito becomes infected, she can later transmit these pathogens to subsequent hosts. But when mosquitoes ingest blood, they must also cope with a variety of stresses, including thermal stress, osmotic stress, and oxidative stress from the digestion of heme-containing proteins (Benoit and Denlinger, 2010; Lahondère and Lazzari, 2012; Saeaue et al., 2011). Oxidative stress can have major impacts on the insect's physiology and metabolism, including affecting its biological clock. For example, it has been shown that behavioral and molecular rhythms in *Drosophila* are redox-sensitive; where flies with a heightened sensitivity to oxidative stress showed a loss of behavioral rhythmicity (Hardeland et al., 2003; Krishnan et al., 2008; Mandilaras and Missirlis, 2012; Zheng et al., 2007). Since flies don't feed on blood and blood-feeding behavior is principal to mosquitoes' role as disease vectors, we hypothesized that diet heme impacts behavioral and molecular rhythms in mosquitoes, which could ultimately impact disease transmission. Despite the inherent need for effective heme and iron metabolism strategies in blood-feeding arthropods, the impact of diet composition on behavioral rhythms remains to be defined. Herein we aimed to determine how diet composition can affect locomotor behavior, and quantified transcript levels of candidate genes that may play a role in host-seeking suppression: *period (per), foraging (for), neuropeptide y-like receptor I (npylr), short neuropeptide F (snpf), catalase (cat),* and *heme oxygenase (ho).*

Ae. aegypti mosquitoes, important vectors of dengue, Zika, yellow fever, and chikungunya viruses, display diurnal locomotor and flight activity patterns, with peaks at the start and end of the day (Eilerts et al., 2018; Jones, 1981; Kawada et al., 2005; Lima-Camara, 2010; Taylor and Jones, 1969). These activity patterns are also dependent on mosquitoes' physiological state and are, for example, altered by insemination, blood-feeding, and infection status (Evans et al., 2009; Gentile et al., 2013; Lima-Camara et al., 2011; Lima-Camara et al., 2014; Luz et al., 2011). In both *Ae. aegypti* and *Anopheles* mosquitoes, blood meal ingestion causes distention of the abdomen and initiates development of the ovaries, which suppresses host-seeking and locomotor activity (Klowden and Lea, 1979; Lima-Camara et al., 2014; Takken et al., 2001).

In addition to proteins that bind and transport odor volatiles, neuromodulators play an important role in regulating host-seeking behavior and olfactory sensory thresholds can also be affected by feeding status (Christ et al., 2017; Duvall et al., 2019; Fadda et al., 2019; Naccarati et al., 2012). Previous studies suggest that neuropeptide Y related signaling plays a role in this regulation (Brown et al., 1994; Duvall et al., 2019; Liesch et al., 2013).

Specifically, neuropeptide y-like receptor 7 (NPYLR7) has been identified as a key member of this signaling pathway and necessary for host-seeking suppression in *Ae. aegypti* female mosquitoes (Duvall et al., 2019). However, the specific mechanism of this phenomenon is unknown; it's possible that peptides activating NPYLR7 may be released from midgut cells and released into the hemolymph, or that blood digestion triggers peptide release directly in the midgut, or are released by neurosecretory cells in the brain (Duvall et al., 2019).

Head peptide-I is a humoral factor that likely plays a role in mediating long-term host-seeking suppression: head peptide-I titers in the hemolymph increase during host-seeking inhibition and injection of head peptide-I into unfed female mosquitoes inhibits host-seeking behaviors (Brown et al., 1994; Matsumoto et al., 1989; Naccarati et al., 2012). Short neuropeptide-F peptides (sNPFs) show sequence similarity to head peptide-I, signal through NPYLRs, and also inhibit host-seeking when injected into unfed females (Liesch et al., 2013; Mertens et al., 2002).

Protein kinase G (PKG) activity is also associated with behavioral modulations in *Drosophila* as well as bees and ants (Ben-Shahar, 2005; Kaun et al., 2007; Lucas and Sokolowski, 2009; Osborne et al., 1997). PKG activity encoded by the *foraging* (*for)* gene is associated with increased foraging behavior in many insects, but little is known regarding the impact on mosquito foraging behavior. However, a potential *for* gene was identified in mosquitoes based on homology to *Drosophila* and mammalian *for,* and it has been shown that *Ae. aegypti* mosquitoes treated with a PKG activator have increased flight and locomotor activity (Keating et al., 2013; Nene et al., 2007). PKG activation is also associated with the night time flight behavior typical in *An. gambiae* mosquitoes (Keating et al., 2013). FOR gene expression encoded has also been found to be rhythmic *D. melanogaster*, as well as *An. gambiae* and *Ae. aegypti* mosquitoes (Ceriani et al., 2002; Keating et al., 2013; Ptitsyn et al., 2011; Rund et al., 2011). Mosquito-borne flaviviruses have conserved PKG phosphorylation sites and *Ae. aegypti* PKG is known to phosphorylate a dengue virus protein (dengue nonstructural protein 5; NS5) and autophosphorylates its regulatory domain (Keating et al., 2013). It's possible that PKG may therefore play a role in causing the increased flight behavior in dengue-infected mosquitoes (Keating et al., 2013; Lima-Camara et al., 2011). Few studies have been conducted on the *for* gene of mosquitoes or other hematophagous insects, and its specific role in modulating host-seeking and nectar-feeding, as well as whether it plays a role in host-seeking suppression following a blood meal, remain unknown.

Evidence in flies shows that heightened sensitivity to oxidative stress also disrupts behavioral rhythms (Zheng et al., 2007). However, the impact of oxidative stress on mosquitoes remains unknown; this knowledge gap has implications in disease transmission, since blood-feeding is essential for their survival and reproduction, as well as the mode of viral transmission to humans. While it's known that blood-feeding decreases locomotion and host-seeking in *Ae. aegypti*, the extent to which ingested heme-containing proteins in the blood contribute to host-seeking suppression, or impact the central biological clock, is unknown (Lima-Camara et al., 2014). While heme and iron are necessary for some proteins and signaling pathways, excess free heme can be toxic, as it can produce reactive oxygen species (ROS) such as superoxide or hydroxyl radicals via the Fenton reaction, disrupt the lipid bilayer in cells due to its lipophilic nature, and lead to cell death (Chaitanya et al., 2016). Several mechanisms protect mosquitoes from oxidative stress. The midgut peritrophic matrix, an extracellular structure composed of chitin, glycoproteins, and proteoglycans, aids in the protection of the midgut from toxic heme and other pathogens (Whiten et al., 2018). Additionally, several antioxidant enzymes are induced by blood-feeding and exhibit synergistic effects (Oliver and Brooke, 2016). For example, superoxide dismutases catalyze the dismutation of superoxide radicals to produce hydrogen peroxide, and catalase is an antioxidant enzyme that converts hydrogen peroxide into water and oxygen. Glutathione S-transferase (GST) is a detoxification enzyme that can bind hematin, and is also implicated in insecticide resistance mechanisms (Saeaue et al., 2011). Heme oxygenase is an enzyme conserved across plants, mammals, and insects that catalyzes the degradation of heme to liberate ferrous iron and produce biliverdin and carbon monoxide (Wilks and Heinzl, 2014). Additional studies have also identified a unique heme degradation pathway involving heme oxygenase in *Ae. aegypti*, whereby its activity is followed by the addition of two glutamine amino acid residues to the heme degradation product biliverdin (Pereira et al., 2007).

We hypothesize that locomotor activity suppression following blood-feeding is modulated by several of the mechanisms discussed herein, both centrally and in peripheral tissues. Therefore, we quantified the expression of a panel of relevant genes in blood-fed and non-blood-fed mosquitoes, as well as mosquitoes fed on a blood mimic diet, either with or without hemoglobin, to determine if changes in behavior and gene expression are impacted by heme-protein digestion.

5.3 Materials and Methods

Mosquito Rearing

Aedes aegypti Liverpool (LVP-IB12, MR4, ATCC®, Manassas, VA, USA) larvae were raised in 26 × 35 × 4 cm covered trays filled with ~1 cm of deionized water, at a density of approximately 200 larvae per tray. They were maintained under light:dark (LD) cycles of 12 h:12 h at 26 °C and 70 ± 10% humidity. Larvae were maintained on daily feedings of Hikari Tropic First Bites (Petco, San Diego, CA, USA). Pupae were isolated on the day of pupation and placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA).

Test Diets

We used an artificial blood meal replacement diet (Gonzales et al., 2018) to explore the role of heme-containing proteins in decreased activity after blood-feeding. For all experiments, mosquitoes were fed during their normal host-seeking peak, *i.e.*, the last two hours of their photophase (ZT 10–12), on one of four test diets: a 10% sucrose solution, heparinized bovine blood, an artificial protein diet containing hemoglobin (modified from (Gonzales et al., 2018)), and an artificial protein diet without hemoglobin. The two artificial diets were prepared by dissolving components in an alkaline phosphate buffer according to (Gonzales et al., 2018) (see Table 5.1 for final concentrations). Artificial diets will henceforth be referred to as "Hb diet" or "noHb diet" to differentiate the two artificial protein diets by the presence or absence of hemoglobin in the diet, respectively. Both unfed mosquitoes and those that were visibly fully engorged were randomly selected and pooled in groups of 5 individuals and weighed to compare the amount ingested of each diet; we found no significant difference in the mass of mosquitoes following ingestion of each of the diets (unfed mass = 1.63 ± .09 mg, blood-fed mass = 3.51 ± .14 mg, Hb-fed mass = 3.94 ± .39 mg, noHb-fed mass= 3.97 ± .27 mg, n = 3–6, N = 15–29, p > .1, Wilcoxon test).

Table 5.1. Components and final concentrations of the Hb and noHb diets. Diets were modified from (Gonzales et al., 2018).

Components	Hb	noHb
Bovine Hemoglobin	10 mg/mL	0 mg/mL
Bovine Serum Albumin	200 mg/mL	210 mg/mL
Chicken Yolk	5 mg/mL	5 mg/mL
Glucose	50 mM	
Adenosine Triphosphate	3 mM	
Sodium Chloride	150 mM	
Sodium Bicarbonate	23 mM	
Potassium Chloride	2.5 mM	
Calcium Chloride	4 mM	
Magnesium Chloride	0.8 mM	

The Hb and noHb diets contain the same total protein mass, with removed hemoglobin from the noHb diet replaced by excess bovine serum albumin (BSA), as shown in Table 5.1. Amino acid composition of all *Bos taurus* (bovine) hemoglobin subunits was compared to that of bovine BSA to confirm that all amino acids present in hemoglobin would still be available in BSA. Indeed, we found that amino acid frequencies were similar, and actually slightly higher in BSA for critical amino acids such isoleucine and proline. Isoleucine is essential for oogenesis and reproductive, while proline supports post-blood meal physiology, ammonia detoxification, and energy production (Briegel, 1985; Scaraffia et al., 2005; Scaraffia et al., 2008; Scaraffia et al., 2003; Rivera-Pérez et al., 2017).

Locomotor Activity

Locomotor and flight activity of female mosquitoes were recorded using a locomotor activity monitor, or actometer (Trikinetics LAM25, Waltham, MA, USA). Six to 8 day-old individual female mosquitoes that fed on one of the four test diets were placed in Pyrex glass cylindrical tubes (25 mm diameter x 125 mm length, Trikinetics, Waltham, MA, USA) immediately after feeding on one of the test diets. Tubes containing individualized mosquitoes were then placed in the activity monitor, which has three infrared beams and detectors per tube opening that bisects the approximate center of the tubes. For the duration of the experiments, mosquitoes were provided with access to 10% sucrose delivered on a soaked cotton ball placed at one end of the tube. With the exception of the sugar fed treatment, only visibly engorged mosquitoes were used for activity experiments. Mosquitoes were maintained in the activity monitor for a duration of 5 days. Reduction in activity after blood-feeding over the 5 days was greatest in the first two days, as expected since eggs are typically ready to be oviposited by three days post-feeding, so our later analysis focused on those two days. At the end of this period, egg deposition in the tubes was visually determined for each individual and, if eggs were observed, the number of eggs present was recorded. Daily locomotion was recorded as the number of beam crossing per 10 min interval. All recordings occurred in a light-proof enclosure with its own lighting system, which consisted of a light-emitting diode (LED) light (800 Lumen, Philips, Amsterdam, The Netherlands) timed to a 12 h:12 h LD cycle aligned to the light cycle of the mosquitoes' rearing conditions. Locomotor activity was quantified over 2 consecutive days starting at the onset of lights on the day following feeding and placement into the individualized tubes. The mean activity was quantified by calculating the William's mean, a modified geometric mean to account for zero values (Haddow, 1960). Individuals that were found dead at the end of the experiments were discarded from calculations if their last time point recorded as active was prior to the end of the two days of activity recording.

Oviposition and Egg Viability Assays

Mosquitoes aged 7–9 days were starved for 24 h and then provided blood, the Hb diet, or the noHb diet, warmed to 37 ℃ via a glass artificial membrane feeder (Lillie Glassblowers, Smyrna, GA). Females that were visibility engorged after feeding were anesthetized on ice briefly until inactive and then individualized into clear polystyrene Drosophila vials (25 x 95 mm, Genesee Scientific, San Diego, CA). Vials were prepared with a small water-laden cotton piece (about 1 in) at the bottom, covered with a round piece of germination paper cut to the vial diameter and enclosed with mesh. Mosquitoes were provided a 10% sucrose-soaked cotton ball placed over the mesh, which was replaced daily. Individualized mosquitoes were maintained under light:dark (LD) cycles of 12 h:12 h at 26 ±1 °C and 70 ± 10% humidity. Over the course of 9 days, mortality was recorded daily and oviposition was monitored. Egg-laden germination papers were dried at 26 ±1 °C and 70 ± 10% humidity for one week, and egg numbers were counted under a stereomicroscope.

Eggs were hatched in 2 oz plastic souffle cups (WebstaurantStore, Lititz, PA) filled with ~1 cm of deionized water, provided fish food daily (Hikari Tropic First Bites, Petco, San Diego, CA, USA), and maintained under the same LD and humidity conditions. Larvae were counted at 3, 4, 6, and 8 days post-hatching.

Transcript Abundance

Samples were collected by pooling 7–14 female mosquitoes (aged 7–9 days) that were visibility engorged after being provided one of the test diets, warmed to 37 ℃ via a glass artificial feeder (Lillie Glassblowers, Smyrna, GA) during the last two hours of light during a 12 h:12 h light:dark daily light cycle (ZT 10–12). At 4, 12, 24, and 48 hours post-feeding, mosquitoes were decapitated and heads and bodies were flash frozen separately in liquid nitrogen and stored at -70 ℃ for later sample processing (Figure 5.1).

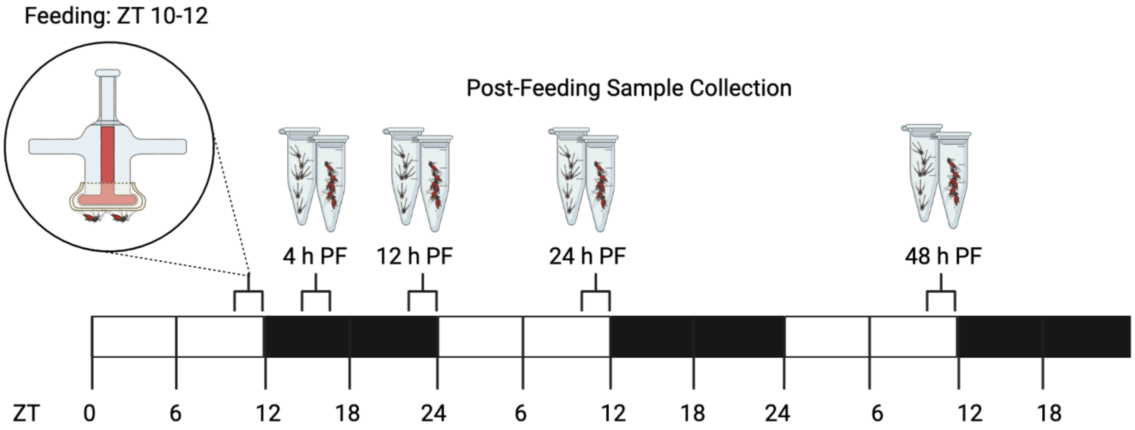

Figure 5.1. Diagram of feeding and sample collection regime for RNA transcript analysis. 7–9 day old mosquitoes were fed on one of the test diets for 1 hour between ZT 10–12. Mosquitoes were pooled and decapitated 4, 12, 24, and 48 hours post-feeding (PF) for later RNA isolation and sequencing.

Total RNA was extracted from each sample using Qiagen RNeasy mini kit and RNase-free DNase (Qiagen, Hilden, Germany) following manufacturer's instructions. Using isolated RNA as a template, complementary DNA (cDNA) was synthesized using SuperScript III first-strand synthesis system (Thermo Fisher Scientific, Waltham, MA). Transcript levels were then quantified by determining fluorescence of SYBR green (Thermo Fisher Scientific, Waltham, MA) of three biological replicates via quantitative real-time PCR using primers specific to each gene of interest (Table 5.2). Each reaction was run with three technical replicates. A non-template negative control was included for each primer reaction. Transcript abundance was determined by calculating the calibrated normalized relative quantities (CNRQ) using the housekeeping 4S ribosomal protein S7 as a reference gene following previously established guidelines (Hellemans and Vandesompele, 2011; Hellemans et al., 2007).

Table 5.2. List of primers used for quantitative PCR. Primers for *npylr* are from Liesch et al., 2013.

Gene	Orientation	Sequence (5'-3')
per	Fwd	GGAGATGAACCCACAGCTAA
	Rev	CGGTAGAGCACAATGTAACTT
for	Fwd	GATAATCCAACGGAGCGACT
	Rev	GAAGGCTGCGATTCCGTA
npylr	Fwd	GCTATCTGCTACATCTGTGTGTCAA
	Rev	GTCCGAGTAGAAGTCGTTGCTCAT
snpf	Fwd	CATCCCTGCGGTTACGATT
	Rev	CCGTAGTTGTGGTGCTCTTAT
cat	Fwd	GGTGTTCGATAGCAAAGCGGAA
	Rev	TTGTCCTTCTGGGACTCCTTGAAC
ho	Fwd	ATCATTCGGCGCACGGGAAA
	Rev	ACTCCACTGTCATAAAGGGCGAAA
rps7	Fwd	GGACAAGAACCAGCAGACCACCA
	Rev	GGGCTCCGGGAATTCGAACGTAA

5.4 Results and Discussion

Gut dissection of mosquitoes that consumed blood, the Hb diet, and the noHb diet confirmed that each diet is directed to the midgut, with none of the diet detectable in the crop (Figure 5.2A-C). Only mosquitoes that had a visibly distended abdomen after feeding were used for oviposition, actometer, and transcript quantification experiments. We found no significant difference in the mean individual weight of mosquitoes classified in this manner as engorged for each of the diets (Figure 5.2D). Mosquitoes from each of the test diets (excluding sugar) had deposited eggs while in the actometer tubes. Notably, a significantly higher proportion of mosquitoes used for activity monitoring had laid eggs when fed either artificial protein diet, as compared to bovine blood: more than 42% of mosquitoes that fed on the Hb diet and 47% of mosquitoes that fed on the noHb diet deposited eggs in the activity monitor setup, while only 17% of blood-fed mosquitoes deposited eggs (Figure 5.2E, $X^2 = 30.042$, df = 1, $p < .001$). While more mosquitoes that fed on the artificial diets deposited eggs in the actometer tubes than those that fed on blood, there was no statistically significant difference between the number of eggs deposited (Figure 5.2F, $p < .05$, *t*-test).

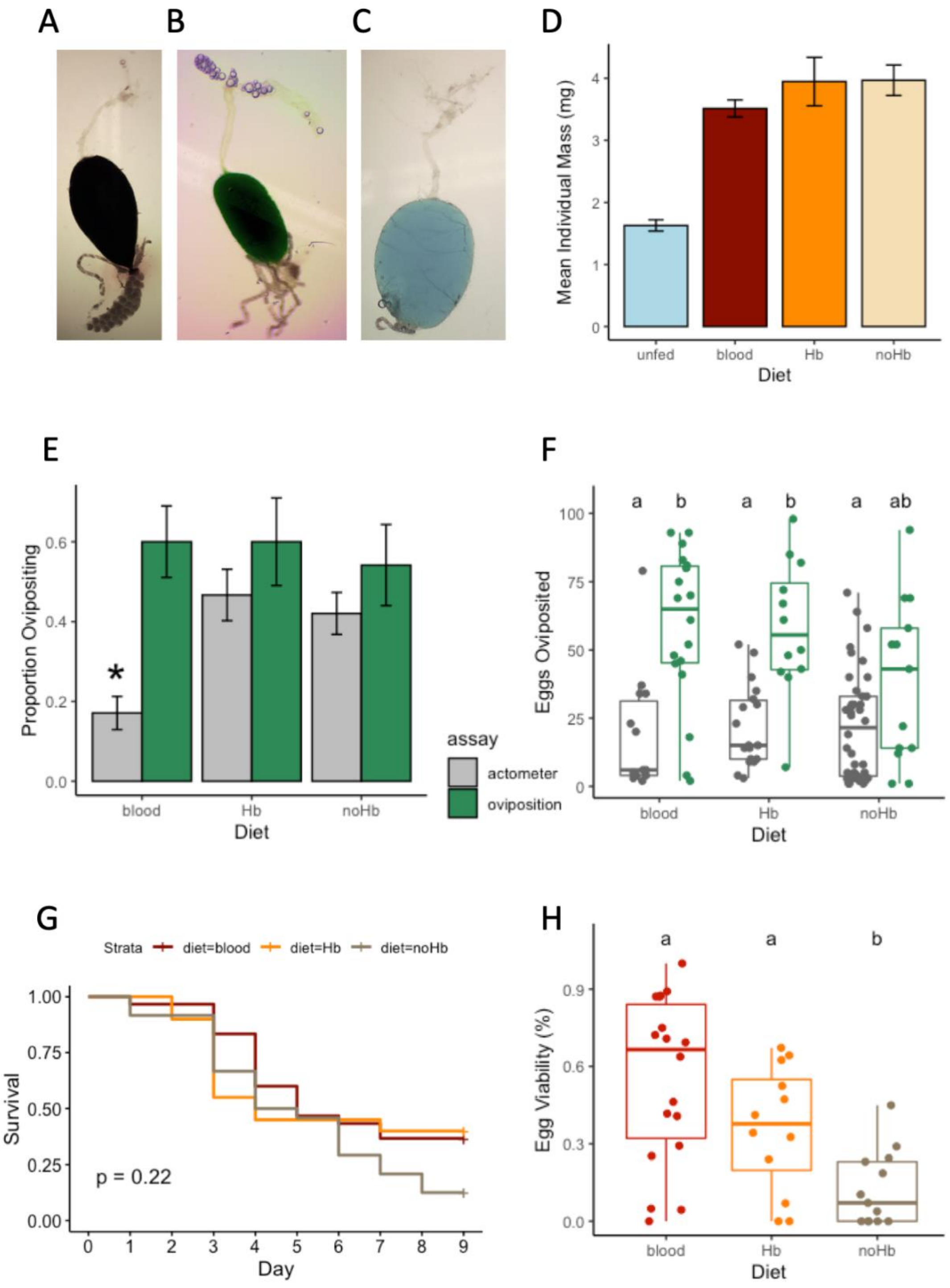

Figure 5.2. Life history traits of *Ae. aegypti* females fed on blood, Hb diet, and noHb diet. Guts dissected of engorged mosquitoes after feeding on A) blood, B) Hb diet (food dye used to enhance visibility of diet in the gut), and C) noHb

diet (with food dye included). D. Mean mass of unfed females and females after feeding on blood, Hb, and noHb diets. Error bars represent standard error of the mean. E. Proportion of mosquitoes ovipositing in the actometer (left, gray) and oviposition assay (right, green) on each of the three diets. Error bars represent standard error of the mean. F. Number of eggs per individual ovipositing in each assay. G. Survival of mosquitoes fed on each of the three diets in the oviposition assay. H. Egg viability of hatched eggs from the oviposition assay.

While this implicates that the artificial diets may produce higher oviposition rates, it's important to note that previous work comparing a similar artificial diet with hemoglobin with bovine blood found no significant differences between the average egg deposition and egg hatch rates (Gonzales et al., 2018). However, these oviposition rates were measured by Gonzales *et al*. by pooling 30 individual engorged females after feeding, while our activity experiments required mosquitoes be individualized in specific tubes (Gonzales et al., 2018). By pooling mosquitoes for their experiments, there may have been differences in the amount of eggs laid per individual that were not identified if a group with less individuals laying eggs actually laid more eggs per individual in those that did oviposit. While the number of eggs oviposited by individuals was not statistically significant, our actometer data do implicate a general trend of increased egg numbers per individual in both artificial diets as compared to blood. However, these oviposition observations were an extension of actometer experiments, as opposed to standard oviposition assays.

In order to determine whether these observations are representative of differing life history traits due to different diets, or simply due to experimental conditions, we quantified oviposition, egg viability, and survival of adult female *Ae. aegypti* mosquitoes after ingesting to engorgement the blood, the Hb diet, or the noHb diet. As compared to the actometer assay, a significantly higher proportion of blood-fed mosquitoes in the oviposition assay laid eggs (Figure 5.2E, $p < .01$, pairwise proportion test). For both the blood-fed and Hb-fed mosquitoes, the number of eggs oviposited per individual was significantly higher in the oviposition assay than in the actometer assay (Figure 5.2F, $p < .05$, *t*-test). While there was no significant difference for noHb-fed mosquitoes for either assay, both oviposition and eggs per individual were higher in the oviposition assay. Therefore, low oviposition numbers in blood-fed mosquitoes used for actometer experiments are likely due to non-ideal oviposition conditions in the actometer assay, such as the preferred oviposition site being near to or on their food source (sucrose-soaked cotton). Mosquitoes fed the Hb and noHb diet may exhibit different metabolic rates or physiological states due to the components in the artificial diets, that may lead them to oviposit more in non-ideal conditions after one meal, as opposed to blood-fed females. There was no significant difference in survival of adult females after consuming each of the test diets (Figure 5.2G, $p = .22$, log-rank test), so differences in oviposition are not likely to be due to general health. Of the eggs recovered from the oviposition assays, eggs oviposited by the noHb-fed mosquitoes hatched at a significantly lower rate (Figure 5.2H, $p < .05$, *t*-test). These results overall indicate that while a protein diet is sufficient for producing viable eggs, hemoglobin in the diet contributes to egg viability. However, researchers have been able to rear multiple consecutive generations of *Ae. albopictus* mosquitoes strictly on a sugar assisted protein diet (containing only 10 or 20 % BSA and 5 % sucrose), and mosquitoes showed egg hatching rates similar to blood-fed mosquitoes

(Suman et al., 2021). Our results may reflect a difference in protein digestion or metabolism between the two species.

Sugar-fed *Ae. aegypti* females show a stereotypical diurnal activity pattern with their largest activity peak at the end of the day (Figure 5.3) while blood-fed females show very low activity across the two test days, with no increase at the beginning of the day and minimal activity increase at the end of the photophase; these observations are consistent with those from established literature (Eilerts et al., 2018; Lima-Camara, 2010; Taylor and Jones, 1969).

Mosquitoes that previously fed on one of artificial diets (with or without hemoglobin) showed overall decreased levels of activity in comparison to sugar-fed mosquitoes (Figure 5.3). Mosquitoes fed the artificial diet containing hemoglobin showed activity patterns similar to that of blood-fed mosquitoes, with slightly higher activity peaks at the end of the day and a visible peak at the start of the second day. They also showed some elevated activity during the second scotophase, which was also observed in blood-fed mosquitoes. Notably, this time corresponds approximately to 60 hours after consuming the diet of interest, so it can be assumed that proteins would be fully digested (Isoe et al., 2009; Li et al., 2019). Additionally, *Ae. aegypti* females are known to oviposit at night despite their typical diurnal behavior and diet composition can impact oviposition and gonotrophic cycle timing (Canyon et al., 1999; Farnesi et al., 2018).

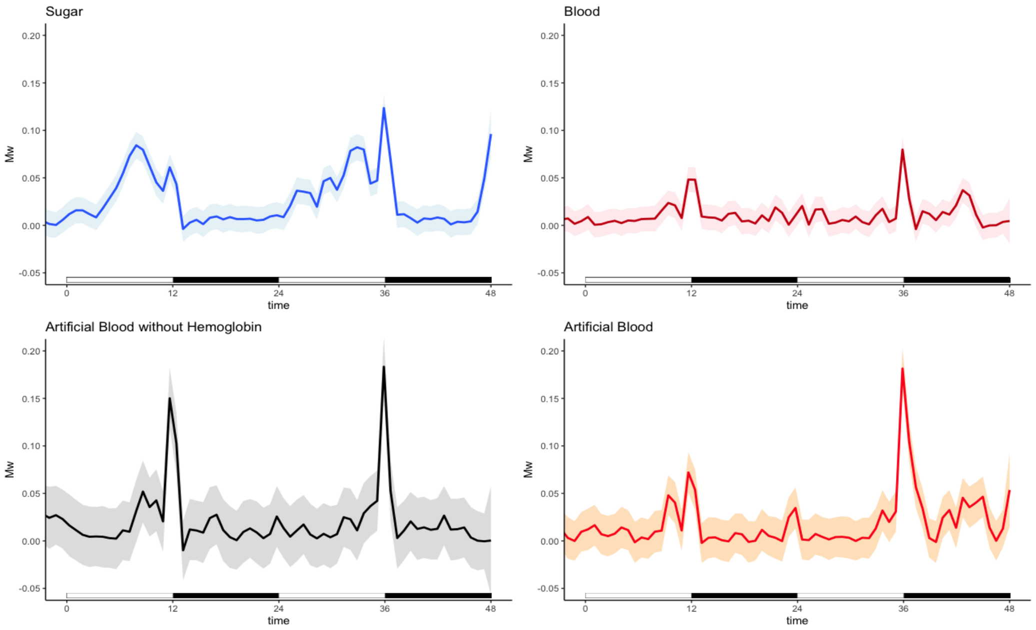

Figure 5.3. Locomotor and activity patterns of female *Ae. aegypti* mosquitoes following ingestion of on of the test diets. Mean activity for two days following ingestion of sugar (blue), blood (red), Hb diet (orange), or noHb diet (black) is depicted as the William's mean (solid lines) and standard error of the mean (shading).

The overall William's mean of activity throughout these two days was statistically significant between diets (F = 9.735, p < .001) and time-of-day (F = 28.544, p < .001) as determined by two-way ANOVA. Mean diurnal activity was statistically different between blood-fed mosquitoes and sugar-fed (p < .001, Tukey post-hoc test), as well as between blood-fed and noHb-fed mosquitoes (p < 0.01, Tukey post-hoc test). Sugar-fed diurnal activity was also significantly different from diurnal activity of Hb-fed mosquitoes (p < .001, Tukey post-hoc test).

In looking specifically at the mean activity of individual mosquitoes during the last 6 hours of the photophase (ZT 6–12; to encompass the majority of their stereotypical sugar-fed activity peak; Figure 5.4), there was a significant difference across groups (Kruskal-Wallis, $p < .001$). Pairwise comparisons revealed significant differences between blood-fed and sugar-fed mosquitoes ($p < .0001$, Wilcoxon test); blood-fed and noHb-fed mosquitoes ($p < .0001$, Wilcoxon test); sugar-fed and Hb-fed mosquitoes ($p < .001$, Wilcoxon test); as well as between the Hb and noHb diets ($p < .001$, Wilcoxon test). Notably, there is no statistical significant difference between blood-fed and Hb-fed mosquitoes during their highest peak of daily activity and there is similarly no significant difference between sugar-fed and noHb-fed mosquitoes at this time.

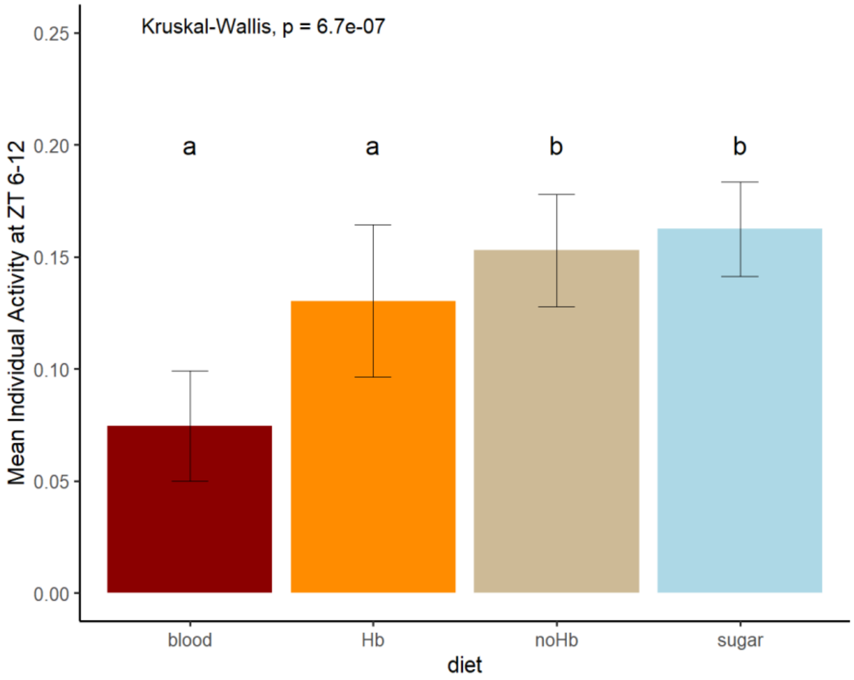

Figure 5.4. Mean individual activity of mosquitoes fed on blood, Hb, noHb, or sugar at ZT 6–12. There was a significant difference across groups (Kruskal-Wallis, $p < .001$). Pairwise comparisons revealed significant differences

between blood-fed and sugar-fed mosquitoes ($p < .0001$, Wilcoxon test); blood-fed and noHb-fed mosquitoes ($p < .0001$, Wilcoxon test); sugar-fed and Hb-fed mosquitoes ($p < .001$, Wilcoxon test); as well as between the Hb and noHb diets ($p < .001$, Wilcoxon test). Bars represent mean activity of individuals and error bars represent the standard error of the mean.

Mosquito head tissue showed a significant decrease in *per* transcript abundance 4 hours after feeding on blood or the Hb diet, as compared to sugar (Figure 5.5A; $p < .05$, *t*-test). This may indicate that hemoglobin or heme released upon heme-protein digestion is responsible for a decrease in *per* transcript abundance, likely leading to decreased PER expression and impacting clock gene expression. While this effect was significant at 4 hours post feeding, there was no significant difference in *per* abundance at 12, 24, or 48 hours post-feeding. It’s possible that *per* transcription is recovered by 12 hours post-feeding, but may also be due to the times chosen for analysis. Specifically, *per* transcript levels cycle throughout the day, and the later times we assessed do not correspond to peak levels, as shown by others and confirmed by our RNAseq data (Chapter 3; (Gentile et al., 2009; Leming et al., 2014)), so it is possible that we would observe a decrease in *per* transcript levels past 4 hours if we assessed a later time point that also coincides with the daily peak in *per* transcript abundance, such as 28 hours post feeding.

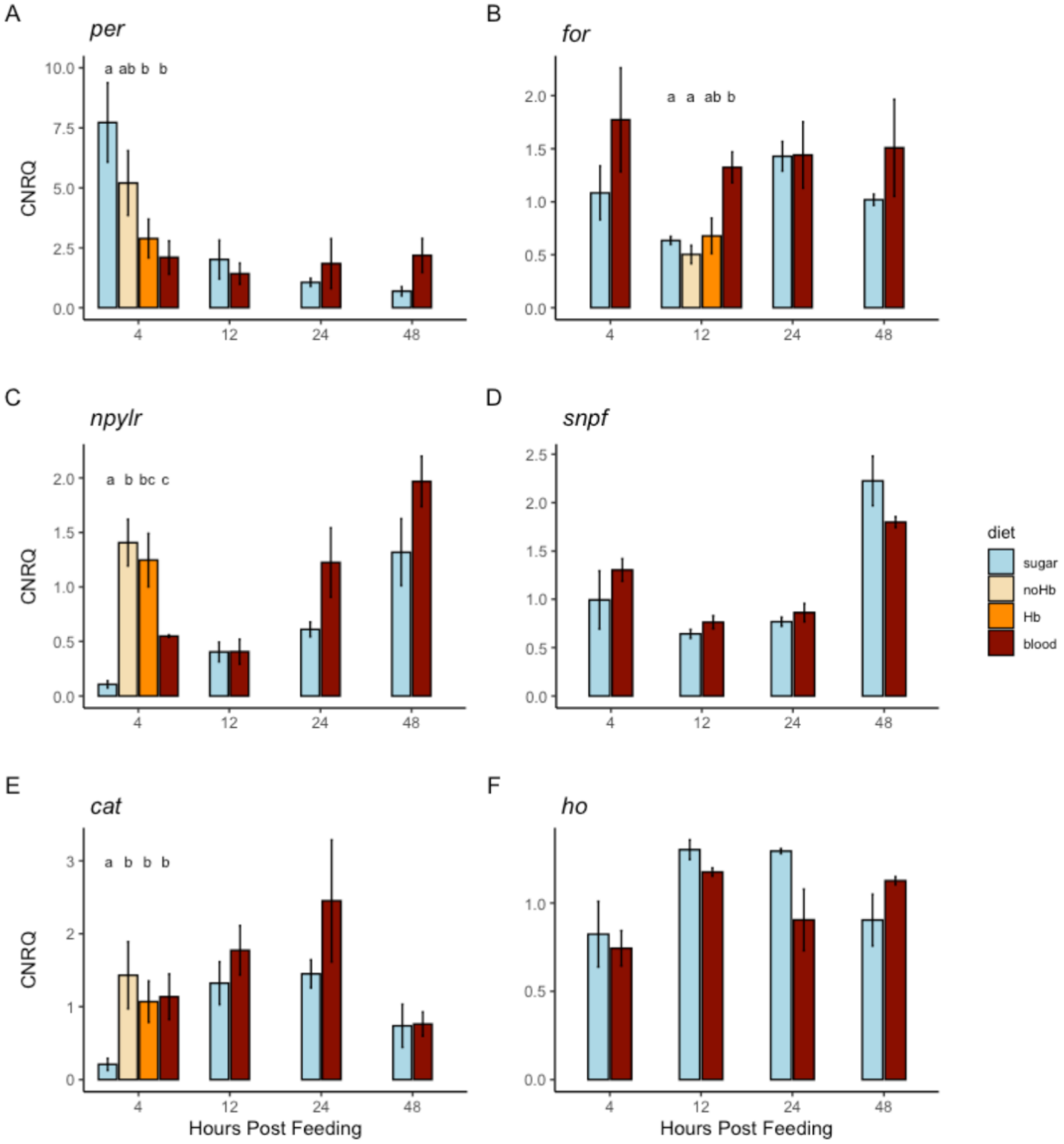

Figure 5.5. Transcript abundance of select genes in female *Ae. aegypti* head tissue. Transcript levels of A) *period*, B) *foraging*, C) *neuropeptide Y-like receptor I*, D) *short neuropeptide F*, E) *catalase*, and F) *heme oxygenase* in mosquito heads after feeding on sugar (blue) or blood (red) diets at 4, 12, 24, or 48 hours post-feeding are shown as calibrated normalized relative quantities (CNRQ) normalized to mean for all samples per gene. When RNA transcript levels between

sugar- and blood-fed mosquitoes at one of the tested times were significantly different ($p < 0.05$, *t*-test performed on log2(CNRQ) values), transcript levels in mosquitoes fed on Hb or noHb diets at the corresponding times post-feeding were also quantified. Error bars represent the standard error of the mean.

In addition to expanding on the tested times, efforts to quantify other clock gene RNA levels may be more informative as they peak at different times, and the effect may only be significant during peak times (Gentile et al., 2009; Leming et al., 2014). Previous research has shown that decreased transcript levels of clock genes in the head 30 hours after blood-feeding at ZT 5 were significant for *period* and *cycle*, but not *timeless* or *clock* (Gentile et al., 2013). Further research to determine whether feeding at different times of day (i.e., ZT 5, during the trough of spontaneous activity, vs. ZT 10–12, peak activity time) leads to differential impacts on clock gene transcription would also be informative, as mosquitoes tend to blood-feed at specific times of day. Expanding on our experiments to include more genes, as well as more times post-feeding would provide insight into the timeline of blood-feeding's impact on clock gene expression.

Head tissue from blood-fed mosquitoes showed a significant increase in *for* transcript levels at 12 hours after feeding when compared to sugar-fed heads (Figure 5.5B; $p < .05$, *t*-test). Increased transcript abundance of *for* just 12 hours after feeding to engorgement on blood is counterintuitive, since *for* is hypothesized to be associated with host-seeking and/or sugar-seeking, neither of which would be likely behaviors 12 hours post blood-feeding to engorgement. However, there is little known regarding the function of FOR in mosquitoes, and it's possible FOR plays a greater role in sugar-seeking than it does in host-seeking. Notably, *for* transcript levels were similar between sugar-fed, Hb-fed, and noHb-fed mosquitoes, so it's possible that *for* transcript levels are lower for those three diets due to the presence of sugar (glucose or sucrose); the artificial diets both contain 50 mM glucose as a phagostimulant, which is about 10 times the concentration of normal bovine or human blood glucose levels (American Diabetes Association, 2018; Holtenius et al., 2000). Therefore, it's possible that the mosquito *foraging* gene modulates sugar-seeking behavior but not host-seeking behaviors.

At 4 hours post-feeding, there was a significant difference between transcript levels of *npylr* in sugar-fed mosquito heads and noHb, Hb, and blood-fed mosquito heads (Figure 5.5C; $p < .05$, *t*-test). Previous research has identified NPYLR1 as a receptor that is sensitive to both Head Peptide-I and sNPFs (Liesch et al., 2013). This research also showed that *npylr* expression is upregulated after blood-feeding, peaking at 48 h post-feeding, which agrees with our observations; however, we also observed an increase in expression over time in sugar-fed mosquitoes, and the difference at each timepoint between sugar-fed and blood-fed mosquitoes was not significant (Liesch et al., 2013).

There was no significant difference between blood-fed and sugar-fed female heads for *snpf* transcript levels at 4, 12, 24, or 48 hours post feeding (Figure 5.5D, $p > .05$, *t*-test). Previous research in *D. melanogaster* showed that sNPFs modulate olfactory perception after fasting to promote food-search behavior; in flies, starvation increases sNPF receptor-1 transcription in olfactory receptor neurons and knocked down sNPF and sNPF receptors in olfactory receptor neurons mimic the fed state in the antennal lobe (homologous to *Ae. aegypti npylr1*) (Root et al., 2011). Additionally, while sNPF was expressed in fed flies, the receptor was only expressed upon starvation and increased sNPF expression was not sufficient to produce starvation-like food-searching behaviors in fed flies (Root et al., 2011). Therefore, it’s possible that the differences between *npylr* and *snpf* transcript levels in fed mosquitoes we observe are due to *npylr* playing a greater role in starvation behaviors than *snpf*; for example, increased *snpf* expression may not affect behaviors if receptors are saturated due to low expression levels. Since mosquitoes used in these assays were visibly engorged, differences in time post-feeding may be reflective of them metabolizing the initial meal over time, and approaching a state closer to that of starvation. Elevated transcript levels in Hb and noHb fed mosquito heads may also be reflective of these diets containing both proteins and glucose.

Blood-feeding in *An. gambia*e has been shown to be under circadian clock control (Das and Dimopoulos, 2008) and it is possible that blood-feeding also feeds back into the circadian clock as a self-regulatory mechanism. It’s unknown whether blood-feeding behaviors are similarly controlled by the clock in *Ae. aegypti*, but since the circadian clock in other organisms has been shown to be redox sensitive, we investigated two oxidative stress management genes in *Ae. aegypti* female heads.

Our results show that *cat* transcript levels are upregulated 4 h after feeding on blood and the Hb diet, as well as the noHb diet (Figure 5.5E, $p < .05$, *t*-test). Since transcript levels were similarly upregulated in the noHb diet as the Hb and blood diets, this initial increase in *cat* transcription following feeding is not due simply to hemoglobin or heme-causing oxidative stress. This may instead be a result of a general stress response due to osmotic stress, heat stress, or protein content. Since these three diets are all ultimately processed in a manner resulting in egg production, they may all cause initiation of similar general signaling processes involved in or related to reproduction. Interestingly, our results show no significant difference between blood-fed and sugar-fed female heads for *ho* transcript levels at 4, 12, 24, or 48 hours post feeding (Figure 5.5F, $p > .05$, *t*-test). In *Drosophila* heads, the rate-limiting enzyme involved in heme degradation, heme oxygenase, cycles and is likely regulated by the clock (Ceriani et al., 2002). Our results agree with previous research showing that *Ae. aegypti* heme oxygenase does not seem to be under a redox-sensitive regulation, as it is in flies (Bottino-Rojas et al., 2019). Since *Ae. aegypti* exhibit this non-canonical transcriptional regulation of heme oxygenase (Bottino-Rojas et al., 2019), in addition to a unique heme degradation pathway (Pereira et al., 2007), this suggests that *Ae. aegypti* have adapted unique mechanisms for heme degradation, which are essential for blood-feeding and their role as disease vectors.

Since heme oxygenase did not show differential transcript levels but *per* transcripts were reduced in mosquitoes feeding on the diets containing hemoglobin, it's possible that the levels of heme surpass the turnover rate of the overall HO present in heads or that *per* transcription is affected by oxidative stress caused by the free iron via the Fenton reaction downstream of heme degradation; this would also coincide with increased *cat* levels that may cope with general redox stress. Overall, our results indicate that redox sensitivity and specifically sensitivity to heme in *Ae. aegypti* heads may be lower due to their adaptation to blood-feeding. Further exploration into the oxidative stress management mechanisms following blood-feeding in *Ae. aegypti* is necessary to understand the unique mechanisms they use to digest and cope with blood stressors.

Body samples from mosquitoes fed on blood showed a significant decrease in *per* transcript levels at 12, 24, and 48 h post-feeding when compared to sugar-fed mosquitoes (Figure 5.6A, $p < .05$, *t*-test). These results suggest that peripheral clocks are more sensitive to longer-term blood-feeding impacts, although further tests would be necessary to quantify the impact in specific tissues such as the midgut. As blood digestion occurs in the midgut, it is understandable that the effects on the clock are more prominent in the body tissue as opposed to the head. However, since *per* transcript levels were similarly reduced in noHb-fed and Hb mosquito bodies, this effect doesn't appear to be driven specifically by hemoglobin or heme ($p < .05$, *t*-test). These results are consistent with a previous study, which similarly showed a stronger impact of blood-feeding on the body at 30 h post-feeding at ZT 5 as opposed to heads in female *Ae. aegypti* mosquitoes (Gentile et al., 2013). That blood-feeding reduces *per* transcript levels in mosquito whole body tissue is especially notable since it's been shown that intracellular ROS impinges on clock gene expression in other organisms (Stangherlin and Reddy, 2013). In *D. melanogaster*, the *per* gene is essential for maintaining antioxidant defense rhythms (Krishnan et al., 2008). Mutant flies lacking *per* showed increased susceptibility to H_2O_2 and decreased catalase activity (Krishnan et al., 2008). It's unclear whether the decrease in *per* transcript abundance in the body is due to a similar upstream decrease in gene expression in the central clock, a downstream modulation of the peripheral clock, or by some other unknown mechanism, perhaps related to the general down regulation of genes following blood-feeding. For example, previous research has shown that transcripts associated with genes involved in transcription and translation decreased in *Ae. aegypti* whole bodies 5 hours post blood-feeding (Bonizzoni et al., 2011).

Similar to our results in head tissue, *for* transcript levels were significantly lower in bodies of sugar-fed mosquitoes as compared to blood-fed mosquitoes at 24 and 48 h post feeding (Figure 5.6B, $p < .05$, *t*-test). However, both the Hb-fed and noHb-fed mosquito bodies were lower than both sugar and blood-fed mosquitoes at 24 h post-feeding and intermediate to sugar and blood-fed mosquitoes at 48 h post-feeding (Figure 5.6B, $p < .05$, *t*-test). Transcript levels of *for* being higher in the bodies of blood-fed mosquitoes than in the other sugar-containing diets is consistent with our results in mosquito head tissue (Figure 5.5), and provides further support to the hypothesis that FOR modulates sugar-seeking behavior but not host-seeking.

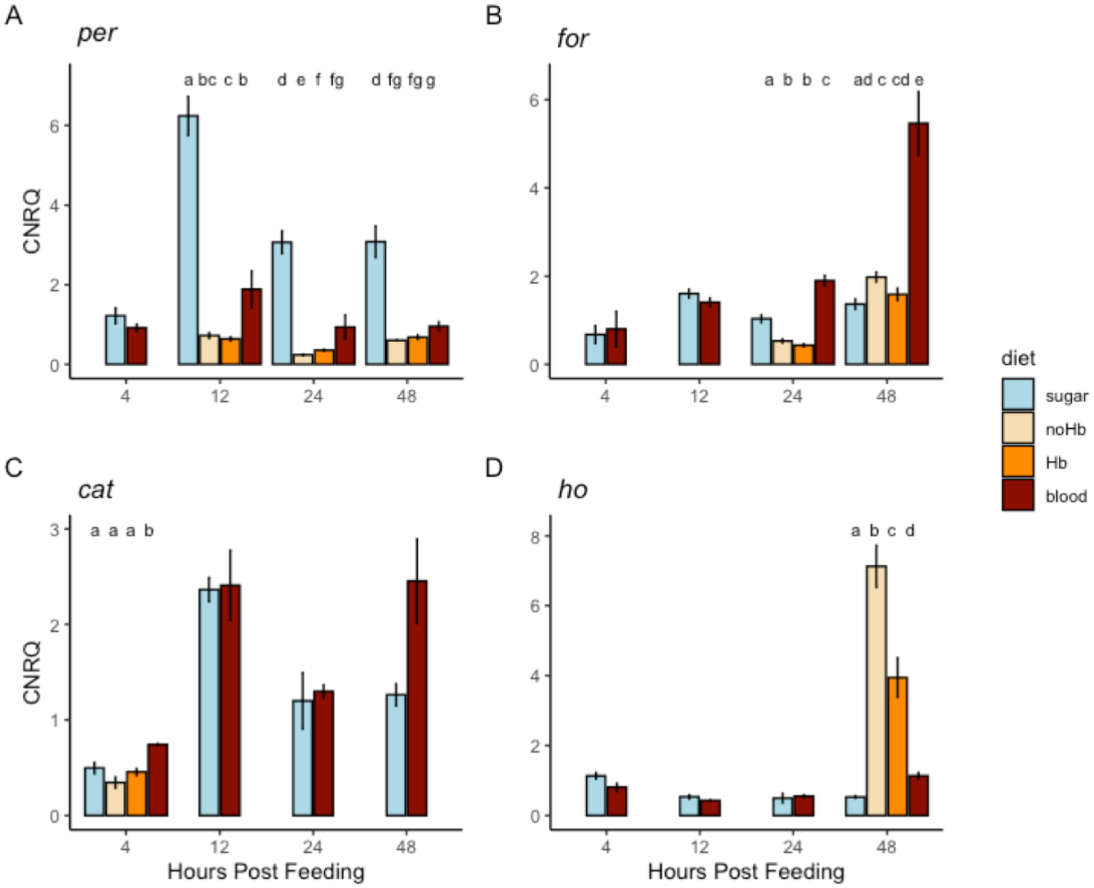

Figure 5.6. Transcript abundance of select genes in female *Ae. aegypti* whole body tissue. Transcript levels of A) *period*, B) *foraging*, C) *catalase*, and D) *heme oxygenase* in mosquito bodies after feeding on sugar (blue) or blood (red) diets at 4, 12, 24, or 48 hours post-feeding are shown as calibrated normalized relative

quantities (CNRQ) normalized to mean for all samples per gene. When RNA transcript levels between sugar- and blood-fed mosquitoes at one of the tested times were significantly different ($p < 0.05$, *t*-test performed on log2(CNRQ) values), transcript levels in mosquitoes fed on Hb or noHb diets at the corresponding times post-feeding were also quantified. Error bars represent the standard error of the mean.

In the body tissue, *cat* transcript abundance was significantly higher in blood-fed mosquitoes as opposed to sugar and both the Hb and noHb diet-fed mosquitoes at 4 hours post-feeding (Figure 5.6C, $p < .05$, *t*-test). This is different from what we observed in head tissue, where *cat* levels in the Hb and noHb diet fed mosquitoes at 4 h post feeding were more similar to blood (Figure 5.5E). Our results suggest that *cat* transcript levels may not be directly linked to heme or even general protein digestion in the body. However, transcript abundance does not reflect the structure or activity of already translated catalase proteins, which could still be affected by cellular oxidative stress. Further studies to determine catalase protein expression and activity may reveal protein-specific impacts of blood-feeding. Interestingly, *ho* transcript levels in blood-fed mosquitoes were significantly higher in the body tissue at 48 h post-feeding (Figure 5.6D, $p < .05$, *t*-test), and subsequent testing of both the Hb and noHb diet-fed mosquitoes at the same time post-feeding showed *ho* transcript abundance was significantly higher than blood-fed mosquitoes (Figure 5.6D, $p < .05$, *t*-test). It's unclear why *ho* transcripts may be highest in the body tissue of mosquitoes that fed on a diet containing no heme proteins 48 hours after feeding. Our results further suggest that, unlike in *Drosophila*, *ho* transcriptional regulation in *Ae. aegypti* is independent of heme concentration and redox state (Bottino-Rojas et al., 2019). Heme oxygenase catalyzes the rate-limiting step in the heme degradation pathway, so it’s particularly interesting that its transcription is not dependent on heme. However, heme degradation also results in the release of free iron, so a dramatic increase in *ho* expression upon blood ingestion could also result in increased ROS production if iron ions are released en masse, so this may actually be advantageous for mosquitoes to have this process unlinked to heme concentration. General blood protein digestion by *Ae. aegypti*, carried out primarily by serine proteases, has been shown to be a complex process involving several proteins that may compensate or work synergistically, regulated via unique mechanisms (Lu et al., 2006; Brackney et al., 2008; Isoe et al., 2009; Eilerts, 2017; Li et al., 2019). Due to the critical role blood digestion plays in the mosquito life cycle, it’s likely that oxidative stress mechanisms downstream of protein digestion exhibit a similar complexity unique from non-hematophagous insects to ensure survival and reproduction following a blood meal.

5.5 Conclusions

Results from the actometer and oviposition assays verified that the actometer setup is not an appropriate method for quantifying oviposition. Oviposition assays showed results more similar to previous studies, and oviposition after feeding once on blood, the Hb diet, or the noHb diet resulted in similar proportions of females ovipositing, as well as similar eggs oviposited per female. However, egg viability was significantly lower in eggs from females fed the protein diet without hemoglobin, suggesting that while hemoglobin may not be necessary for reproduction, it does enhance hatch rates. This may be due to the specific amino acid composition of the protein and heme itself likely plays a role, as iron is important in many signaling pathways and in egg development, as over half of the retained heme from a blood meal is ultimately found in the eggs (Zhou et al., 2007).

Blood-feeding decreases stereotypical *Ae. aegypti* locomotor activity patterns. Hb and noHb-fed mosquitoes exhibit intermediate activity patterns, with Hb-fed mosquitoes showing activity patterns closer to blood-fed mosquitoes, and noHb-fed mosquitoes showing activity patterns closer to sugar-fed mosquitoes, suggesting that hemoglobin plays a specific role in this activity decline. In order to gain insight into the mechanisms at work in decreasing activity after feeding on blood or the Hb diet, we quantified transcript levels of 6 genes in mosquito heads and 4 genes in mosquito bodies 4, 12, 24, and 48 h after feeding on sugar or blood, as well as Hb and noHb if there was a significant difference between sugar and blood-fed mosquitoes for each gene and timepoint. We found that the circadian pacemaker gene *per* showed decreased transcript levels in mosquito heads 4 h after feeding on blood or the Hb diet, indicating that hemoglobin may play a role in decreased clock transcription. In body tissue, *per* transcript abundance was decreased at 12, 24, and 48 h post-feeding in blood as well as both the Hb and noHb diet, so *per* transcript reduction in the body is independent of hemoglobin but not proteins in general. Head tissue *cat* transcript abundance increases following ingestion of blood, Hb diet, and noHb diet, so *cat* transcript levels in the head likely represent a general stress response due to consuming a hot protein meal, as opposed to a specific response to oxidative stress caused by heme. In the body as well, *cat* transcript levels did not seem to be directly linked to protein or heme specifically. Transcript abundance of *npylr* and *snpf* in head following ingestion of these diets suggests that *npylr* may be more important in regulating starvation behaviors in *Ae. aegypti* females. In both head and body tissues, our results also suggested that *for* is likely involved in sugar-seeking but not host-seeking behaviors in *Ae. aegypti* females.

Blood digestion and nutrient utilization by female mosquitoes are critical components of host-seeking suppression. Our results demonstrate that there is likely a complex network of interactions between multiple genes that wasn't captured by focusing on a panel of candidates. Further research is necessary to determine the nuanced systems mosquitoes rely on to cope with oxidative stress and how this may impact their host-seeking and physiology. This line of questioning would benefit from a global transcriptomic approach, such as RNAseq, as well as from focusing on specific tissues such as the midgut or malpighian tubules, to gain a better understanding of these complex processes. By understanding the mechanisms at work, we may be able to exploit these processes to aid in preventing vector-borne disease.

5.6 Author Contributions

Conceptualization, D.F.E. and C.V.; methodology, D.F.E, K.C., and C.V.; formal analysis, D.F.E. and C.V.; investigation, D.F.E., K.C., O.E., M.V., and C.V.; writing—original draft preparation, D.F.E.; writing—review and editing, D.F.E. and C.V.; visualization, D.F.E. and C.V.; supervision, C.V.; project administration, C.V.

CHAPTER 6: SUMMARY AND CONCLUSIONS

6.1 Summary and Future Directions

Rhythms in mosquito biology and behavior mediate many aspects of mosquito-host interactions and, eventually, impact disease epidemiology. In particular, olfactory processes that mosquitoes rely on to find a host are regulated by biological rhythms. Once female mosquitoes successfully find and bite a human host, blood digestion and associated reproductive processes further regulate mosquito activity patterns and host seeking. Overall, results of the studies presented here, show that olfactory rhythms are resource specific, plastic, and mediated by rhythmic gene expression.

Plasticity in mosquitoes activity patterns has been observed here in two different contexts: first, that of odor activation of mosquito locomotor activity. Spontaneous locomotor activity is often used as a means to predict when mosquitoes will be active and a risk for disease transmission. Here we show that the presence of both background concentrations (Chapter 4) and plumes (Chapter 2) of chemical cues, resulted in an activation of mosquito locomotion. Unexpectedly, this effect was the strongest at ZT 0–2, the start of the day. When presented at this time of day and for a limited duration, odor exposure also resulted in a change in mosquitoes’ activity at other times of day.

While we saw that olfactory sensitivity is modulated throughout the day (Chapter 2), transcriptomic analysis only identified a handful of chemosensory genes that were differentially expressed throughout the day (Chapter 3). However, this may be due to limitations associated with sampling, as the transcriptomic analysis here was performed on whole heads and chemosensory appendages (antenna, maxillary palps), which make up a relatively small volume of the total head tissue. Further studies, at the level of the antennae only, are necessary to obtain a more refined comparison of differences in transcript abundance between specific OBPs and ORs. Additionally, olfactory behavior may be modulated at cognitive levels as opposed to sensory processing. Based on our findings that rhythms in antennal sensitivity to odors are not synchronized with rhythms in the behavioral responses to odors, it's likely that olfactory behavior is modulated at both the central and peripheral levels.

The second context that plasticity of activity patterns has been observed is following a blood meal, where (as shown by others) the general locomotor activity is reduced after the ingestion of a blood meal. However, here we used artificial diets that allowed us to decouple the contribution of blood-meal induced oxidative stress from hemoglobin. By removing hemoglobin from an artificial blood-mimic, we found that oxidative stress from blood likely decreases transcription of central clock genes immediately following ingestion, but that mosquitoes can compensate for this effect within 12 hours of feeding. While the central clock is relatively robust in response to oxidative stress, we found that peripheral tissues in the rest of the body show decreased levels of clock gene transcription following a protein meal, but this was independent of hemoglobin. This, in combination with transcript quantification of two oxidative stress management genes, *heme oxygenase* and *catalase*, suggest that the central clock may be redox sensitive but peripheral clocks are not; however, our results indicate that mosquitoes likely exhibit a general physiological stress response after ingestion of a hot protein meal, regardless of hemoglobin inclusion. This is likely reflective of mosquitoes' adaptations to blood-feeding and increased redox tolerance. Overall, our results indicated redox homeostasis following a blood meal is complex and divergent from *Drosophila*, likely as a result of adaptations to cope with blood-feeding. Future research into other genes involved in stress responses and exploration into the effects of oxidative stress on the clock, including other clock transcription factors, will be informative. Additionally, since transcript levels do not reflect protein levels or functionality, research aimed at investigating the impact of oxidative stress on redox enzyme activity may provide insight into the mechanism behind decreased clock transcription following a blood meal.

RNA transcript analysis herein also suggests that *neuropeptide y-like receptor I* transcription plays a more important role in regulation *Ae. aeygpti* starvation behaviors than its ligand, *short neuropeptide F.* Additionally, the role of the *foraging* gene, which regulates sugar-seeking and foraging behaviors in flies and other insects, in mosquito sugar- and host-seeking has remained ambiguous. Our results here suggest that *for* is involved in sugar-seeking behaviors but not host-seeking in *Ae. aegypti* females. The role of these genes can also be further explored by evaluating their expression under starvation conditions. This work focused on the induced response to feeding at the end of the day (ZT 10–12), but it remains unknown how these genes may be expressed throughout the day constitutively, and whether basal rhythms in their gene expression, both centrally and in peripheral tissues, impact mosquitoes' ability to cope with blood ingestion at different times of day. It would also be of interest to compare the induced response in mosquitoes feeding in off-peak times of day.

We focused our efforts on diurnal rhythms but it remains unclear if these are maintained by the central clock or by external light cues only. This is of particular interest as domesticated anthropogenic *Ae. aegypti* live in urban areas and are typically exposed to artificial light at night, which can disrupt or alter some behavioral rhythms. By characterizing the circadian nature of these rhythms, we can better address how to target the mechanisms at work. Additionally, longer rhythms such as lunar and seasonal rhythms in behaviors and physiology are likely very important to vector biology and ultimately disease transmission. The potential for rhythms in oxidative stress management also implies additional rhythms in immunity and detoxification processes, both of which are critical in determining mosquito susceptibility to viral infection and insecticides. Future research to determine how time of day and circadian rhythms mediate mosquito physiology in this context is likely to provide important insight into effective or improved methods to address disease transmission.

Another aspect of mosquito rhythms that warrants further investigation is their sleep patterns, as locomotor/flight activity and likely their sleep/wake cycles impact their physiology as well as vectorial capacity (Ajayi et al., 2020). Vector-host interactions likely result in divergent sleep patterns from non-hematophagous insects, as mosquito behaviors must accommodate the rhythms of their host for reproduction. Very few sleep studies in mosquitoes have been performed despite its relevance to circadian biology, host-seeking and host-biting behaviors, and immunity.

By providing an increased understanding of the molecular and physiological mechanisms involved in host-seeking and blood meal processing we can gain insight into the central modulation of host-seeking behavior. Such processes represent targets of opportunity for vector control strategies, and a stepping stone to solve the inadequacy of current prevention and treatment options required to successfully mitigate vector-borne diseases. Overall, this work can be leveraged in future research to identify potential targets to disrupt mosquito-host interactions.

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