Regulation of Juvenile Hormone Synthesis by 20-Hydroxyecdysone in the Yellow-fever Mosquito, Aedes aegypti

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REGULATION OF JUVENILE HORMONE SYNTHESIS BY 20-HYDROXYECDYSONE IN THE YELLOW-FEVER MOSQUITO, *Aedes aegypti*

A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BIOLOGY by Maria Areiza 2018
To: Dean Michael R. Heithaus  
College of Arts, Sciences and Education

This dissertation, written by Maria Areiza, and Regulation of Juvenile Hormone Synthesis by 20-Hydroxyecdysone in the Yellow-fever Mosquito, *Aedes aegypti*, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: May 31, 2018

The dissertation of Maria Areiza is approved.

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Andrés G. Gil  
Vice President for Research and Economic Development  
and Dean of the University Graduate School

Florida International University, 2018
DEDICATION

I dedicate this dissertation to my two special guys: my best friend and husband Andrew and my little man James. Your love is my guiding North Star that gave me the strength to complete this journey. Thank you, Andrew, for always believing in my crazy dreams, despite how serpentine the path. Also, I would be remiss if I did not mention to my four-legged kids who never let me take myself too seriously; Byron and Monkey you were faithful friends wished you would have been there at the end. And last but not definitely not least, to my family who without their unwavering support I would not have crossed the finish line. Thank you, Ingrid you are the village, that every woman needs.
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I owe a debt of gratitude to my committee members, Dr. Matthew DeGennaro, Dr. Marten J. Edwards, Dr. Lidia Kos, and Dr. Kalai Mathee, for their support and advice. Special thanks to Dr. Matthew DeGennaro who introduced me into the world of transgenic mosquitoes – your help, wit, and support can never be thanked enough.

Lastly, a very special thank you to all the mosquitos I worked with during this project, they gave the ultimate sacrifice for science, their lives. This work was made possible by funding MBRS: NIH/NIGMS R25 GM061347
ABSTRACT OF THE DISSERTATION

REGULATION OF JUVENILE HORMONE SYNTHESIS BY 20-HYDROXYECDYSONE IN THE YELLOW-FEVER MOSQUITO, Aedes aegypti

by

Maria Areiza

Florida International University, 2018

Miami, Florida

Professor Fernando G. Noriega, Major Professor

In Aedes aegypti, development and reproduction are regulated by juvenile hormone III (JH) and the ecdysteroid 20-hydroxyecdysone (20E). While their individual roles as endocrine regulators of development and reproduction are well characterized, the plausible interendocrine control of synthesis between these hormones is not fully understood. Juvenile hormone is synthesized by the corpora allata (CA), a pair of endocrine glands with neural connections to the brain. Circulating JH titers are largely determined by the rate of biosynthetic activity of the CA which is tightly regulated by allatostatic and allatotropic factors. In this study, we evaluated the role of 20E in the activation of the late pupal CA and in the reactivation of JH synthesis post blood meal (PBM). Remarkably, in vitro stimulation with 20E of the CA of the early pupa (24h prior to eclosion or -24h) prematurely initiated JH synthesis at a time when transcript levels for most JH biosynthetic enzymes are low. Moreover, the application of 20E correlated with an increase in the enzymatic activity of juvenile hormone acid
methyltransferase (JHAMT), a critical enzyme of the biosynthetic pathway. Additionally, separation of the CA from the brain increased JH synthesis. Together, these results indicate that 20E acts as a developmental mediator of CA maturation which overrides an inhibitory effect of the brain. For A. aegypti, a blood-meal is required to complete vitellogenesis and results in suppression of CA activity. However, the CA must be reactivated to initiate the second gonotrophic cycle. Our findings show that in vitro stimulation with 20E at 24h PBM reactivates the gland. Stimulation with 20E in increased activity of another key enzyme, farnesal dehydrogenase (FALDH). These results suggest a stimulatory role of 20E on the biosynthetic activity of the CA in the blood fed female. To further elucidate the role of 20E on JH biosynthesis in the adult mosquito the CRISPR/Cas9 system was used to begin to establish an inducible CA specific transgene expression system. The findings of this study constitute the first robust evidence that 20E play an allotropic role in CA activity via the activation of key JH biosynthetic enzymes.
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**LIST OF ABBREVIATIONS AND ACRONYMS**

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<th>Description</th>
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<tr>
<td>20E</td>
<td>20-hydroxyecdysone</td>
</tr>
<tr>
<td>BF</td>
<td>Blood-Fed</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic Helix-Loop-Helix</td>
</tr>
<tr>
<td>BR</td>
<td>Brain</td>
</tr>
<tr>
<td>CA</td>
<td>Corpora Allata</td>
</tr>
<tr>
<td>CC</td>
<td>Corpora Cardiaca</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>ETH</td>
<td>Ecdysis Triggering Hormone</td>
</tr>
<tr>
<td>ETHRs</td>
<td>Ecdysis Triggering Hormone Receptors</td>
</tr>
<tr>
<td>FA</td>
<td>Farnesoic Acid</td>
</tr>
<tr>
<td>FAL</td>
<td>Farnesal</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl Diphosphate</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-Protein-coupled Receptors</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>JH</td>
<td>Juvenile Hormone</td>
</tr>
<tr>
<td>JHBPs</td>
<td>Juvenile Hormone Binding Proteins</td>
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<tr>
<td>JHs</td>
<td>Juvenile Hormones</td>
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<tr>
<td>MF</td>
<td>Methyl Farnesoate</td>
</tr>
<tr>
<td>MP</td>
<td>Mevalonate Pathway</td>
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<tr>
<td>PGs</td>
<td>Prothoracic Glands</td>
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</table>
PBM Post blood meal
PTTH Prothoracicotropic Hormone
q-PCR Quantitative Real Time PCR
RNAi RNA Interference
SF Sugar-Fed
TF Transcription Factor
CRISPR clustered regularly interspaced short palindromic repeat-associated endonucleases
VM Ventromedial Cells
YFP Yellow Fluorescent Protein
Chapter: 1 Introduction

“Human beings can easily destroy every elephant on earth, but we are helpless against the mosquito.”

Asimov

1.1 Geographical distribution and vector expansion of Aedes aegypti

Aedes aegypti, also known as the ‘yellow fever mosquito’, is a highly anthropophilic mosquito that thrives in densely populated areas. It is the primary arthropod vector of several globally dispersed human arboviruses. Native to Africa, A. aegypti is now distributed worldwide in both tropical and subtropical regions which include parts of the continental United States (Christophers, 2009). Historically, its geographic range has been limited, in part, by its intolerance to cold temperatures, to the regions between the northern January and southern July 10 °C isotherms (Figure 1). However, in the past 25 years there has been an increase in its global distribution; raising the potential of re-emerging arboviruses being introduced to naïve populations (Jansen and Beebe, 2010). A recent study from the CDC reported occurrences of the mosquito in 25 U.S. states including the District of Columbia from 1995-2016 (Hahn et al., 2016). For instance, while A. aegypti is typically found from the Gulf coast to the southeast, Mid-Atlantic, and New York, more recently it has become established in the Southwest (Eisen and Moore 2013, Kraemer et al. 2015, Porse et al. 2015, Lima et al. 2016). Along with increased urbanization, the scaling back of control efforts, and future global climate warming - there is a possibility of a continued expansion of the
geographic ranges of vector species like mosquitoes. A well-documented example of this phenomenon is the northward range expansion of *Ixodes*, the primary tick vector of the Lyme disease. Recent decades have seen an expansion in the tick's northern geographical range from the United States into southern Canada, Northern Sweden, and to higher altitudes in central Europe (Materna et al. 2008, Jaenson et al. 2012, Leighton et al. 2012). From a public health perspective, the possibility of range expansion of *A. aegypti* is especially troubling because of the potential for the transmission of pathogens by infected mosquitoes.

1.2 The domestication of *Aedes aegypti*: the evolution of an urban pest

One aspect that has important implications for disease transmission of mosquito associated arboviruses is the adaptability of *A. aegypti* to human habitation. The domestication or human commensalism of *A. aegypti* is the result of an evolutionary adaptation to specialize in feeding from humans (Powell et al., 2013; Brown et al. 2014, McBride et al., 2014). Its presumed ancestral form was African *Aedes formosus*; a sylvan mosquito that lived in sub-Saharan Africa. This mosquito likely would oviposit in natural containers such as tree holes and was zoophilic (generalist) (Mattingly 1957, 1967; Christophers, 2009; Moore et al., 2013). In contrast, the domestic form of *A. aegypti* has evolved to breed in peri-domestic and domestic environments and is anthropophilic (specialized) (McBride et al., 2014). Unlike sylvan mosquitoes, *A. aegypti* adult females are attracted by human odor and will readily enter houses and lay eggs close to human populations in artificial containers such as flower pots, water tanks, and
tires (Gouck, 1972, McBride et al., 2014). The eggs which are laid above the water line can resist desiccation for up to a year until water is available for them to hatch (Shannon and Putnam, 1934; Christophers, 2009; Soares-Pinheiro et al., 2017). But perhaps one of the most troubling adaptations of A. aegypti that increases risk of disease transmission is its feeding preference. Even in the presence of other hosts, female mosquitoes will feed preferentially and frequently from human hosts during each gonadotropic cycle (Harrington et al., 2001; De Benedictis et al., 2003; Scott et al., 1993).

1.3 Emerging arboviruses: Dengue, Zika, Chikungunya, and Yellow Fever

Indeed, the selective and frequent feeding on humans by A. aegypti is of import as this mosquito is the primary vector of four significant viral diseases: dengue fever, Zika, Chikungunya, and yellow-fever. Alarmingly, outbreaks of urban epidemics for all of these diseases have been on the rise worldwide (Jansen and Beebe, 2010; Gardner and Rydman, 2010; Gould et al., 2017). The resurgence by A. aegypti in areas where the mosquito had once been eradicated has been linked to the reemergence of these arboviruses.

While the origin of dengue is unclear, it has been posited to have originated from either Asia or Africa about 1,000 years ago (Mackenzie et al., 2004). Dengue is caused by four serotypes (DENV1-4) and sequential infections with different serotypes increase the risk for a more serious condition, known as dengue hemorrhagic fever (DHF). Each year, the World Health Organization (WHO) estimates that there are 390 million DF infections. Of which, 500,000 of
them progress to DHF and result in 22,000 deaths, mostly of children (WHO, 2017; CDC, 2017). Currently 40% of the world’s population is at risk of DF making it the most prevalent of mosquito-borne diseases (Bhatt et al., 2013). While outbreaks of DF have occurred throughout history they had been limited in geographical scope. In fact, the first documented epidemic of DHF was in 1953 in Manila, Philippines (Gubler, 2011). And prior to the 1970s only nine countries had outbreaks of DHF, but now it is endemic in over 100 countries (Gething et al., 2012; Brathwaite et al., 2012). In October of 2012, Europe saw its first dengue epidemic since the 1920s with 1,891 cases (Seixas et al., 2013). Notably, this dengue outbreak in Madeira Island (Portugal) was autochthonous and raises concerns for a spread of dengue into continental Europe (Sousa et al., 2012). In the American tropics, the Aedes aegypti eradication program initiated by Pan American Health Organization (PAHO) had effectively controlled outbreaks of dengue fever (DF) and yellow fever (YF) (Gubler et al., 1995; Gubler and Wilson, 2005; Gubler, 2008). However, once vector control programs were discontinued in the early 1970s the mosquito was quickly reestablished. By 1981, Caribbean and Latin American regions that had been free of DF for more than 35 years, reported outbreaks of (DHF) (Pinheiro, 1989; CDC). While most documented cases in the United States are associated with international travel, there have been incidences of locally acquired outbreaks in Texas (2005), Hawaii (2001), and Florida (2013) (Bouri et al., 2012, CDC; WHO). In fact, the first locally acquired cases of Dengue in the continental United States (outside of the Texas-Mexico border) since 1945 were in Florida dating back to August 2009 (CDC,
The virus is now endemic in Puerto Rico, the U.S. Virgin Islands, and in Samoa and Guam raising the specter for the reemergence of Dengue in the U.S. in years to come.

Another arbovirus that has more recently emerged in the Americas is the Zika virus (ZIKV). Zika was first isolated from rhesus monkeys in 1947 in the Zika forest of Uganda and isolated in humans in 1952 (Dick et al., 1952). Until recently, Zika was virtually an unknown tropical disease with outbreaks in Africa and Southeast Asia. However, this all changed in 2007 with the emergence of Zika in the Yap islands of Federated States of Micronesia (Duffy et al. 2009). In this large outbreak, over 70% percent of the island inhabitants were infected. The virus then spread into four other pacific islands including French Polynesia in 2013-2014 which is thought to be source of the emergence of ZIKV in the Americas (Pettersson et al., 2016). Remarkably, this outbreak of ZIKV was the first time that the virus was associated with microcephaly and Guillain-Barré syndrome (Oehler et al., 2014; Musso and Gubler, 2016). By 2015 microcephaly case clusters were being reported in Brazil leading the WHO to declare ZIKA a public health emergency of international concern (Gulland, 2016). Since then, Zika has spread throughout the Americas, with local mosquito borne transmission occurring in 47 countries and territories (PHAO, 2017; WHO, 2017). In 2016, the CDC reported 5,102 cases of Zika in the U.S. (224 presumed autochthonous) and 36,079 in U.S. territories (35,937 presumed autochthonous) (CDC, 2016). One of the most striking aspects of the epidemiology of ZIKV is the
possibility for non-vector-borne transmission which has been associated with the virus in the form of: intrauterine mother-to-child, sexual, and post-transfusion transmission (Grischott et al., 2016).

After more than 30 years of absence, another mosquito-borne virus to have reemerged is the Chikungunya virus (CHIKV). While historical accounts point to epidemics taking place as early as 1823-1824 in Zanzibar and India (Halstead, 2015); the virus was only first isolated in 1952 from a patient during an outbreak in modern day Tanzania (Ross, 1956). The name of the disease is derived from the Makonde dialect which translates to "that which bends up" or "walking bent over" or referring to the posture of patients which present severe joint pain (Robinson, 1955). Since then, subsequent outbreaks of the virus in the 1960s and 1970s had been limited to Africa, Southeast Asia, and the Indian subcontinent (WHO, 2017). However, in 2004 the coast of Kenya experienced two major epidemics of CHIKV; first in Lamu Island (June) and then in Mombasa (November) (Sergon et al., 2004; Chretien et al., 2007; Staples et al., 2009). Here, a seroprevalence survey estimated that 75% of the population of Lamu was infected, that is 13,500 infections from a population of 18,000 (Sergon et al., 2004; Chretien et al., 2007). Genetic characterization of this virus, indicated that the strain known as, Indian Ocean lineage (IOL), then spread eastward in a large scale epidemic that struck the islands of the Indian Ocean and India proper in 2005-2006 (Njenga et al., 2008). These outbreaks were particularly striking both in their geographic expansion and their extent. For instance, in La Reunion alone
approximately 255,000 cases were reported and in Comoro 65% of the population became infected (Sergon et al., 2005; Josseran et al., 2006). Similarly, India officially reported 1.39 million infections in over 16 states (WHO, 2007). By 2007, Chikungunya emerged in Europe for the first time. The first locally acquired transmissions were reported in Italy in August of 2007; followed by outbreaks in France in 2010 and 2014 (Rezza et al., 2007; Grandadam et al., 2011; Delisle et al., 2014). Nine years after the first outbreaks in Kenya, CHIKV emerged on the islands of the Caribbean. In December of 2013 autochthonous cases of Chikungunya were reported in the French sector of the Caribbean island of St. Martin and then quickly spread throughout the Americas (Fischer et al., 2014). By 2014 the virus had arrived in the continental United States. Though most infections were associated in travelers returning from affected areas (2,811) Florida had 12 locally transmitted infections (Kendrick et al., 2014). According to the CDC, there were 4,659 autochthonous infections in the U.S. territories of: Puerto Rico, the U.S. Virgin Islands, and American Samoa. Currently, the PAHO reports that local transmission of Chikungunya occurs in 45 countries and territories in the Americas.

Lastly, yellow fever continues to be a significant public health concern worldwide, especially for Africa and the Americas. Presently, endemic transmission of yellow fever virus (YFV) occurs in tropical and subtropical areas of Africa and South America. The WHO estimates that YF is endemic and intermittently epidemic in 47 countries of Sub-Saharan Africa and 13 countries in
the Americas. Yellow fever burden estimates are hard to compile as cases are often underreported (Gardner and Rydman, 2010). But it is estimated that there are 130,000 cases of YF and 78,000 deaths annually (Garske et al., 2014).

Yellow fever is in fact an old disease; it is believed to have originated in Africa approximately 3,000 years ago. It was first introduced to the Americas in the 1600s by slave ships coming from West Africa (Cathey and Marr, 2014). The first recorded case is thought to have occurred in Yucatan in 1648 (Carter, 1931). Since then, periodic outbreaks have occurred in the 17th, 18th, 19th, and early part of 20th centuries (Gubler, 2004). The last major YFV epidemic in United States took place in New Orleans in 1905 in which there were 3,402 cases and 452 deaths (Tomlinson and Hodgson, 2005). In the Americas overall, the last major outbreak took place in Brazil in 1942 (Monath, 1988). However, by the 1940s, mosquito eradication programs in the Americas and the mass vaccination programs in Africa contributed to control urban epidemics of YFV (Gubler, 2004; Gardner et al., 2010). Unfortunately, in the following decades as these efforts waned, the risk of large outbreaks increased.

The virus has three transmission cycles: sylvatic, intermediate, and urban. First discovered in 1935, the sylvatic or “jungle cycle” is one of the facets that make eradication of YF a challenge. In the sylvatic cycle, the virus is maintained by lower primates and canopy dwelling mosquitoes. Human transmission is incidental and happens when individuals enter the jungle; as in the case of occupational or recreational activities. The intermediate transmission cycle the
most common type of outbreak in rural villages of the Africa savannah (Gardner et al., 2010). Intermediate transmission involves semi-domestic mosquito species that infect both humans and monkeys. In contrast, in the urban cycle transmission occurs between humans as the primary reservoir of the virus and A. aegypti as the main vector.

While most YF infections in both Africa and the Americas have been associated with sylvatic and intermediate cycles, there have been some significant outbreaks that raise concerns. More recently, in the Americas during late 2007 and early 2008 a cluster of cases were reported in Brazil, Argentina, and Paraguay in close to areas infested with A. aegypti. In Paraguay, these autochthonous YF infections were the first in 34 years with a total of 28 confirmed cases and 9 deaths (PAHO). Most remarkably, the infections in Asuncion, Paraguay, were shown to be urban acquired – these being the first cases in 34 years with a total of 28 confirmed cases and 9 deaths (PAHO). Most remarkably, in Asunción the cases were shown to be urban acquired infections. While the scope of this YF outbreak was small, it was the first outbreak of urban YFV in the Americas after a 65 year absence. Moreover, with the reestablishment of A. aegypti in most of its pre-eradication range, it raises concerns that YF urban epidemics can reemerge in the Americas. Likewise, Africa saw an emergence of one of the largest outbreaks of urban yellow fever in over 30 years. In December of 2015, Angola experienced a large outbreak in its capital and largest city, Luanda. The epidemic subsequently spread to all of the
18 provinces of the country and lasted until October of 2016 leaving behind 884 confirmed cases including 78 fatalities (WHO; Ortiz-Martínez et al., 2017; Paules and Fauci, 2017). Ultimately the outbreak extended to the neighboring Democratic Republic of Congo where 78 cases were reported of which 16 cases resulted in deaths (WHO). In response to the outbreak, a preventive mass vaccination campaign went into effect which was able to control the epidemic. However, the campaign highlighted one of the central fears that YF epidemics could overwhelm vaccine stockpiles. Indeed, during this outbreak the global emergency stockpile reserved for epidemic response was exhausted, prompting the WHO to adopt fractional dosing to extend vaccine supply (Wu et al., 2016). In addition, since 2010 YFV activity has been increasingly shifting from East Africa towards the West in regions where mass vaccination campaigns are lacking; and given the rapid urbanization of Africa there is potential for large-scale urban outbreaks (WHO potential).

Presently, existing treatment options are limited as therapeutics are not available against these mosquito-borne diseases and a vaccine exists only for yellow fever. As a result, vector control is critical in attempting to reduce viral infections in susceptible populations. Compounding the issue is the emergence of insecticide resistance among mosquitoes. For instance, a major challenge for vector control programs targeting A. aegypti mosquitoes find mosquitoes with mutations that render them resistant to common insecticides such as: pyrethroids and dichloro-diphenyl-trichloroethane (DDT) (Soderlund & Knipple 2003; Vontas
et al., 2012). These challenges underscore the necessity to better understand mosquito biology in order to generate effective and targeted vector control strategies. An attractive target for mosquito vector control is the master regulatory hormone juvenile hormone III (JH).

1.4 Dissertation Objectives and Organization

In *A. aegypti*, JH is a key hormonal regulator of development, metamorphosis, and reproduction. Juvenile hormone is synthesized by a pair of specialized endocrine glands with neural connections to the brain called the *corpora allata* (CA). Because JH is not stored in the CA but rather is released as it is synthesized, circulating JH titers are largely determined by the rate of biosynthetic activity of the gland (Li *et al.*, 2003). In turn, CA activity is tightly regulated by allatostatic (inhibitory) and allatotropic (stimulatory) factors. Regulation of JH biosynthesis is a complex process that is mediated by factors that are linked to both developmental and nutritional signals (Pérez-Hedo *et al.*, 2013; Noriega, 2014; Nouzova *et al.*, 2015; Areiza *et al.*, 2015). These include neuropeptides, a diverse class of signaling molecules which in insects, have pleiotropic roles as both neuromodulators and neurohormones (Nässel, 2002). For instance, allatotropin (AT) and allatostatin (AS) are two examples of allatoregulatory neuropeptides of insects (Kataoka, 1989; Woodhead, 1989). First isolated from the adult moth *Manduca sexta*, allatotropins are known to be stimulatory to JH synthesis and have been characterized in *A. aegypti* (Kataoka, 1989; Li *et al.*, 2004; Nouzova *et al.*, 2012). Conversely, allatostatins have been
demonstrated to have an inhibitory effect in JH synthesis and have also been described in *A. aegypti* (Hernández-Martínez *et al.*, 2007).

Indeed, the CA of *A. aegypti* expresses receptors for various putative regulatory molecules such as allatotropins, allatostatins, ecdysis triggering hormone (ETH), and the insect ecdysteroid 20-hydroxyecdysone (20E) (Nouzova *et al.*, 2012; Areiza *et al.*, 2014). Ecdysis triggering hormone is a neuropeptide well-established for its role in the orchestration of ecdysis or molts in insects. Recently we have demonstrated that ETH has a dual function in the last hours of the pupal stage: Namely, it orchestrates ecdysal behavioral motor sequences and synchronizes the reactivation of the pupal CA (Areiza *et al.*, 2014). Thus, ETH makes the gland competent to synthesize JH in the 6-8 hours prior to eclosion. Similarly, the allatotropic qualities of ETH have also been observed in *Drosophila melanogaster* where it has been shown to maintain JH levels that are critical in ensuring reproductive success in adults (Meiselman *et al.*, 2017; Lee *et al.*, 2017). Notably, both ETH and its cognate receptors are under direct transcriptional control of the 20E (Park *et al.*, 2003; Zitnan *et al.*, 2002, 2003; Zitnan and Adams, 2012; Shlyueva *et al.*, 2014). In this context, 20E acts as an upstream signal that is indirectly able to activate CA biosynthetic activity presumably via ETH. Moreover, the expression of 20E receptors in the CA lends possibility to the idea that it too can act as an allatoregulatory player.

Also known as the ‘molting hormone,’ 20E is critical regulatory hormone of insect development and reproduction that has been proposed as a modulator
of JH synthesis in the lepidopteran tobacco hornworm, *M. sexta*, and the silk-worm *Bombyx mori* (Whisenton *et al.*, 1985, 1987; Rankin *et al.*, 1986; Gu and Chow, 1996; Kaneko *et al.*, 2011). However, in mosquitos (Diptera) it was unknown if 20E regulates JH biosynthesis. Notably, a recent study in *D. melanogaster* has revealed cross-talk between 20E and JH in the regulation of metamorphosis, as a result of interendocrine regulation of biosynthesis (Liu, *et al.*, 2018). The goal of the current project was to elucidate the possible role of 20E in the activation of the late pupal CA and in the reactivation of the CA in the blood-fed female. Biosynthetic activity of the CA is regulated by an integration of factors that are linked to developmental and nutritional signals; the work in the dissertation demonstrates that 20E plays a role in both aspects.

The findings in the dissertation advance our understanding of the physiological significance of regulatory points in the JH biosynthetic pathway. Chapter 2 reviews important aspects of mosquito endocrinology and describes the known roles of JH and 20E in the mosquito life cycle with emphasis on their roles in development and reproductive biology. Chapter 3 reviews molecular genetic approaches of transgenic manipulation in *A. aegypti*. Chapter 4 is a reprint of a previously published article in *Insect Biochemistry and Molecular Biology* which shows our findings of 20E as a developmental mediator of CA maturation in the late pupal stage. Lastly, Chapter 5 examines the effect of 20E on the reactivation of the CA post blood meal in preparation for the second gonadotrophic cycle.
In summary, mosquito-borne diseases are a major health concern worldwide causing significant human suffering and mortality. *Aedes aegypti* is an important vector of such diseases including dengue fever, Zika, Chikungunya, and yellow fever. As a hematophagous insect, the female *A. aegypti* mosquito requires a blood meal for egg development. Transmission of mosquito-borne diseases takes place when a female mosquito takes a blood meal from a viraemic host and then feeds from another non-viraemic host. The selective and frequent feeding on humans by *A. aegypti* magnifies the risk of transmission of these arboviruses. Hormonal regulation of reproduction by (JH) is a critical component of female fitness, and therefore of vectorial capacity. As such, interference of JH synthesis can be an attractive means for vector control. The biosynthetic activity of the CA is affected by many factors. Our research is revealing the importance of previously unknown developmental JH modulators playing distinctive roles during the different physiological states of the CA. The work of this dissertation attempts to address the process that allows for a switch from an inactive an active CA and will furthermore add to our knowledge of mosquito reproductive biology.
Figure 1. World Distribution of *Aedes aegypti*. Contour lines depict the January and July isotherms defined by the limits of geographical range of northern and southern hemisphere year-round survival.
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Chapter 2: A review of Juvenile Hormone and 20-hydroxyecdysone

2.1 Introduction

Throughout the life history of insects, hormones regulate most aspects of their existence as they undergo striking changes in development and physiology. The importance of hormone regulation is especially true for holometabolous insects like mosquitoes which have distinct developmental events such as larval molts, pupation, and eclosion into the sexually mature adult. In insects, endocrine control is a complex process that is largely mediated by two different classes of hormones, neurohormones and true hormones. Neurohormones, or neuropeptide hormones, are produced by specialized neurosecretory cells that are found throughout the nervous system (Nijhout, 1994). While true hormones are synthesized by two specialized glandular tissues; such as the corpora allata which synthesizes juvenile hormones and the prothoracic glands (PGs) and gonads, which synthesize ecdysteroids (Riddiford, 1980).

Together, juvenile hormones and ecdysteroids are important endocrine regulators of a host of physiological, developmental, behavioral, and reproductive events. For instance, in immature insects, JH acts in conjunction with the ecdysteroid hormone 20-hydroxyecdysone (20E) to control metamorphosis. Here the periodic pulses of ecdysteroids are responsible for the initiation of molts while JH dictates the nature of the molts (Riddiford, 1994; Gilbert et al., 2000). In the adult, the hormonal interplay between JH and 20E continues as both hormones regulate various aspects of its life cycle including reproductive maturation,
nutrient sensing, and behavior. Remarkably, in insects, hormones that regulate development have been repurposed to control aspects of reproduction in the adult. This is the case with JH and 20E which, in the mosquito, are responsible for mediating the vitellogenic process (Riddiford, 1994; Raikhel, et al., 2002).

While their individual roles as endocrine regulators of development and reproduction are well characterized, the plausible interendocrine control of synthesis between these hormones is not fully understood. For example, in *Aedes aegypti*, existing temporal relationships between 20E peaks and JH biosynthesis hint at an interaction that may extend beyond their regulatory functions. The aim of the present project was to elucidate how 20E might act as a regulatory effector of JH biosynthesis. The chapter serves as an introduction of these two hormones, their historical perspective, biosynthesis, and mode of action and will provide a comprehensive background to the work of the dissertation.

### 2.2 A brief history of the discovery of Juvenile Hormone and 20-hydroxyecdysone

The notion that hormones play a morphogenetic role in insects began in the early part of the 20th century with seminal works from Stefan Kopec and Vincent Wigglesworth (Kopec, 1917;1922, Wigglesworth, 1934). Their pioneering work led to the discovery of the hormonal control of molting and metamorphosis by JH and 20E and laid the foundation for modern insect endocrinology. Interestingly, while it was assumed that as lipid soluble hormones, JH and 20E would exert their molecular basis of action via nuclear receptors, their
mechanism of action remained elusive for some time (Oro et al., 1990; Yao et al., 1992; Koelle et al., 1991; Charles et al., 2011). Since then much has been gleaned on their pleiotropic effects including their function as gonadotropins in the reproductive process (Wigglesworth, 1936; Wyatt; 1991; Bownes, 1986; Koepppe et al., 1985). It is in this context that the following sections aim at giving a background of these two endocrine partners with emphasis in their roles in molting and vitellogenesis.

2.2.1 Discovery of Juvenile Hormone: the long road

The groundwork for the discovery of juvenile hormone began with anatomical and histological work undertaken by various researchers throughout the 19th century. This body of work led to the description of the corpora allata (CA), the endocrine glands that synthesize and release JH (Cassier, 1979; Gilbert et al., 2000). First reported by Müller in 1829 in the cockroach, the CA were described as pharyngeal bodies that he believed to be part of the stomatogastric nervous system (Müller, 1828). Because their role was unknown, the glands were designated various names such as the corpora incertae when they were identified in ant and the ganglia allata in the embryo of Forficula auricularia (earwig) (Meinert, 1861; Heymons, 1895). And it was not until 1899 that Heymons coined the term, corpora allata in a paper that described their structure and embryological origin in the walking stick, Bacillus rosii (Heymons, 1899; Cassier, 1976; Gilbert et al., 2000).
However, the endocrine function of the CA would be first described in 1913 by Nabert, who had worked with several groups of insect orders. He identified the CA as an endocrine gland of unknown function that exhibited periodic internal secretion (Nabert, 1913). This would be further corroborated by Ito in 1918 through histological studies on several species of Lepidoptera where the CA was found to be a functioning gland in the adult moth (Ito, 1918). Though the endocrine nature of the CA had been reported, the actual physiological significance that it played in molting and metamorphosis remained elusive. The first glimpses that hormones regulated this process were shown by Stefan Kopec’s work on larva-pupal molt of the gypsy moth, *Lymantria dispar* (Kopec, 1917; 1922). After employing simple surgical techniques like ligation of larvae and decapitation, Kopec concluded that a factor from the brain was critical in the initiation of molts.

Building on Kopec’s notion, Vincent Wigglesworth began his studies in the 1930s to understand the endocrine factors that were mediating molting. Using the blood-sucking bug, *Rhodnius prolixus*, he provided a mechanistic answer to the question of how metamorphosis was taking place in insects. Also known as the “kissing bug” as a result of their penchant for biting humans around the mouth or eye, *R. prolixus* is a triatomite bug found throughout Latin America which is the primary vector of the Chagas disease parasite, *Trypanosoma cruzi* (Bargues et al., 2010). As a hemimetabolous insect, *Rhodnius prolixus* undergoes “incomplete” or simple metamorphosis, without ever entering a pupal
stage. Upon hatching into a nymph or a larva, it undergoes a gradual change in body form with five successive molts into nymphaal stages (instars) prior to metamorphosis into the adult form (Uribe, 1926). *Rhodnius prolixus* proved to be a good model animal for Wigglesworth, as the nymph molts take place in response to a large blood meal which allowed him to establish a critical period of molting. By performing several surgical procedures, he identified both the source and timing of hormonal control of molts. Using the same methods that Kopec had previously used, he was able to corroborate the endocrine role of the brain and called the molt eliciting factor a “molting hormone” (Wigglesworth, 1934). In particular, he found that when decapitation took place prior to the critical period, there was an arrest in development. In his surgical studies he used a technique known as parabiosis, in which a developmentally arrested animal was surgically joined with a normally developing one and the circulatory systems merged. He observed that a circulating hormone in young nymphs prevented a fifth (last) stage nymph from progressing to the adult stage, leading him to believe that a second factor was preventing the expression of adult characteristics. Through histological work he suggested that the CA could be playing the source of what he called the “inhibitory factor” (Wigglesworth, 1936). By 1940 he coined the name “juvenile hormone” and defined the morphogenetic role of JH with emphasis on its action at the cellular level (Wigglesworth, 1940). As the name implies, JH has an antimetamorphic function that promotes the retention of juvenile characteristics. In the following years analogous experiments were
performed on other insects of various taxa that would validate Wigglesworth’s findings (Bounhiol, 1938; Piepho, 1943).

One of the main hurdles facing researches studying this new hormone was the difficulty in obtaining large amounts of JH from the CA. Indeed, earlier attempts to extract significant amounts of JH extracts had proven difficult and hindered studies on its action. These barriers were all but removed by work from Carroll Williams on the silk moth, *Hyalophora cecropia*. Williams is credited with launching the modern era of JH research with his serendipitous discovery of large amounts of soluble JH in the abdomen of male adult moths (Williams, 1956; Williams, 1959). Originally, he had sought to prolong the life of the adult moths by conducting a parabiosis with a pupa. What he found instead was that the pupa molted into another pupal stage, demonstrating that the adult moth was the source of JH. Here, the abdomens contained a store of a lipoidal JH which, after extraction, yielded a dark orange material he called “golden oil.” This led the way for the purification of the hormone and ultimately the structural identification of JH.

Using the lipid extracts from the silk moth, Roller and colleagues were the first to determine the chemical structure of JH-I (Roller et al., 1967). The lab of Howard Scheniderman would expand on this work in the silk moth by corroborating the structure of JH-I and identifying another homologue, JH-II (Meyer et al., 1968). Several years later, JH-III was discovered when it was isolated from organ cultures of CA from the tobacco hornworm moth, *Manduca*.
sexta (Judy et al., 1973). The homologue JH-III is the most ubiquitous naturally occurring juvenile hormone found in insects, including A. aegypti. Additional JH structures have been identified in the years following, JH-0, 4-methyl JH-I, JHB3, and JHSB3. The first two, JH-0 and 4-methyl JH-I (iso-JH0) were found in the eggs of the tobacco hornworm (Bergot et al., 1981). JHB3, also known as JH III bisepoxy, was isolated from in vitro culture of ring glands of Drosophila melanogaster (Richard et al., 1989). Lastly, JHSB3 is a skipped bisepoxide which was identified in the brown-winged green bug, Plautia stali (Kotaki et al., 2009). A common feature of all the JH homologues which have been characterized to date is their farnesoid structure (Figure 2) (Noriega, 2014; Adams, 2009; Goodman and Cusson, 2012). Moreover, there are JH analogs which are non-insect derived compounds that possess JH-like biological activity in insects. Examples are of these are fenoxycarb, pyriproxifen, and methoprene (Figure 2). As a consequence of their exceptional biological activity in insects they have been used commercially as a control agent for insect pests, including mosquitos, since 1975 (Goodman and Cusson, 2012; Riddiford, 2012).

Naturally occurring juvenile hormones (JHs) are comprised of a family of acyclic sesquiterpenoids that are, by in large, limited to insects. To date, eight JH homologues have been identified in approximately 100 insect species, encompassing 10 insect orders (Noriega, 2014; Goodman et al., 2012). Interestingly, methyl farnesoate (MF), which is the immediate precursor of JH in A. aegypti has also been found in Crustacea where it has been suggested to play
a role in development and reproduction (Homal and Chang, 1997; Borst et al., 1987; Laufer, H. et al., 1987; Hinsch, 1980). Methyl farnesoate is also present in higher Dipterans like in the black blow fly, *Phormia regina* and *D. melanogaster* (Yin et al., 1995; Jones and Jones, 2007; Develiers, 2013). However, despite the diversity of naturally occurring juvenile hormones, in all insects, whether hemimetabolous or holometabolous, JH universally regulates metamorphosis.

The discovery of JH has been a long process that has spanned three centuries. The tools used to study it have evolved in many ways from the histological and microsurgical techniques used by Kopec, Wigglesworth, and Williams in the 19th century and early 20th century. Though limited by the constraints of available technology, the painstaking work of early researchers led to the revelation that molting and metamorphosis were under hormonal control; laying the foundation for modern insect endocrinology. Moreover, the role of JH in determining the developmental outcome of the molt was defined. Today, a combination of microsurgery, biochemistry, molecular biology, and genetic tools attempt to broaden our understanding of this versatile hormone and undoubtedly will lead to more discovery, particularly at the molecular level. Currently, our understanding of the mechanisms which regulate the actual biosynthesis of JH continue to have unanswered questions. It is the goal of this dissertation to elucidate some aspects that regulate JH biosynthesis.
2.2.2 Juvenile Hormone Biosynthesis

Throughout the life history of *A. aegypti* mosquitoes, JH biosynthesis is a dynamic process which is tightly regulated to ensure proper timing of developmental and gonotrophic cycles. Juvenile hormone is synthesized and secreted by the *corpora allata*, a pair of neuroendocrine glands. Located in the thorax and posterior to the brain, the CA are ovoid bodies that are innervated by axons of cerebral origin, (neurosecretory cells and typical) and subesophageal ganglion (Nijhout, 1994; Cassier, 1979). In addition to the brain, the CA is connected by nerve fibers to the *corpora cardiaca* (CC); together they form the retrocerebral complex (Nijhout, 1994). The CC, is a small paired neurohemal organ that produces neurohormones and serves as a storage area for peptide hormones produced in the brain (Goodman and Cusson, 2012). These small discrete glands are approximately 40-50 µm in length and synthesize adipokinetic hormone (Kaufmann *et al.*, 2009; Li *et al.*, 2003). Each corpus cardiacum is made up of only six neurosecretory cells and is fused around the aorta (Clements, 1992). In the female mosquito, the CA is comprised of approximately 48 cells in the pupal stage and 60 in the adult (Clements, 1992). The glands undergo volumetric changes which are coincident with developmental transitions, and in some cases, are associated with increase in secretory activity (Szibbo and Tobe, 1981; Clemens, 1960). Nonetheless, the small size of these endocrine glands poses technical challenges both in surgical manipulation and in the scope of techniques available to study them.
Juvenile hormone biosynthesis in mosquitoes takes place in a series of 13 sequential enzymatic steps which are conventionally divided into early and late steps (Figure 3). The early steps belong to the mevalonate pathway (MVP) in which acetyl-CoA is converted to farnesyl diphosphate (FPP). Here acetyl-CoA is reductively polymerized into five-carbon (5C) isoprenoid units. The isoprene units are then sequentially condensed to form FPP. These steps are conserved in all organisms that use the MVP to synthesize cholesterol or other biomolecules. However, insects and other arthropods have lost the ability to synthesize cholesterol de novo and instead rely on dietary intake as their source (Bellés, et al., 2005). In particular, insects lack the genes encoding enzymes which are needed for the biosynthesis of cholesterol from FPP; among them, squalene synthase. Instead, FPP is used for production of compounds such as ubiquinone, the prenylation of membrane-bound proteins, and for JH synthesis (Goodman and Cusson, 2012; Bellés, et al., 2005). The late steps, are generally considered to be JH-specific and are comprised of five enzymatic steps. In A. aegypti mosquitoes, FPP is transformed sequentially to farnesol (FOL), farnesal (FAL), farnesoic acid (FA), methyl farnesoate (MF), and ultimately JH III. First, FPP is catalyzed by farnesyl pyrophosphatase to FOL. Then, farnesol is sequentially oxidized into FAL and FA. The order of the last two enzymatic reactions in the biosynthetic pathway differ between insect orders (Goodman and Cusson, 2012). For example, in Lepidoptera, epoxidation precedes methyl esterification by juvenile hormone acid methyltransferase (JHAMT). Here, FA is epoxidized by a P450 monooxygenase and converted to JH acid, or JHA. Then
methyl esterification is catalyzed by JHAMT (Reibstein et al., 1976). By contrast, in Orthoptera, Dictyoptera, Coleoptera and Diptera esterification of FA into methyl farnesoate (MF) takes place before epoxidation to JH III (Feyereisen et al., 1981; Defelipe et al., 2011). Ultimately, the biosynthetic activity of the CA is determined by the progression of the JH pathway which is mediated by both the size of precursor pools and enzymatic activities (Nouzova et al., 2011; Rivera-Perez et al., 2014).

The biosynthetic activity of the CA is regulated by allatoregulatory factors which can be stimulatory (allatotropic) or inhibitory (allatostatic) in nature. In A. aegypti females, the glands alternate between four distinct physiological states, which in turn reflect changes of biosynthetic activity. Moreover, these physiological states of CA activity are linked to reproductive physiology and are defined as: inactive, active, modulated, and suppressed (Rivera-Perez et al., 2014; Hernández-Martínez et al., 2015; Zhu and Noriega, 2016). Regulatory signals which control CA biosynthetic output act at three different levels: eliciting cytological, constitutive/long-term, and dynamic/short-term responses (Noriega, 2014; Unnithan et al., 1998; Applebaum et al., 1991). Cytological or “slow responses” are those resulting in gross morphological changes that modulate the physiology and maximal biochemical productivity of the corpora allata (Unnithan et al., 1998; Applebaum et al., 1991; Tobe and Pratt; 1976). These cytological responses, are exemplified by cell number and volumetric changes of the CA associated with development and reproduction (Chiang et al., 1995). Long-term responses result in constitutive changes occurring within several hours to days.
In these types of responses, the spontaneous rate of JH synthesis is easily detected by *in vitro* assays (Unnithan et al., 1998). Examples are variations of biosynthetic enzyme levels and CA changes in responsiveness to allatoregulatory peptides (Li *et al.*, 2003). In comparison, short-term responses take place within the scale of minutes to hours and are typically reversible (Li *et al.*, 2004). Control of JH synthesis by allatostatin and allatotropins are examples of dynamic responses. Taken together, JH synthesis regulation comprise various regulatory mechanisms, including neuronal and neuroendocrine factors.

Correspondingly, the regulatory mechanisms stimulating, and inhibiting CA function are complex, involving an integration of developmental cues and nutritional status. In female mosquitoes, regulation of JH biosynthesis is especially nuanced during the vitellogenic cycle in which the transduction of nutritional signals is critical for oogenesis. Thus, it is not surprising that the CA-CC complex of *A. aegypti* expresses receptors for various regulatory molecules such as allatotropins (AT), allatostatins (AS), insulin, ecdysis triggering hormone (ETH), and 20-hydroxyecdysone (20E) (Figure 4) (Areiza *et al.*, 2014; Nouzova *et al.*, 2012; Mayoral *et al.*, 2010). The effects of these regulatory molecules reflect their roles as either nutritional or developmental modulators of JH synthesis in the life cycle of the mosquito.

Within this framework, allatostatin-C (AST-C) and insulin reflect a nutritionally dependent modulation of JH synthesis in female mosquitoes; while ETH and 20E are developmental regulators (Perez-Hedo *et al.*, 2013; Areiza *et
Both AST-C and insulin exert their effects by controlling the availability of precursors but not necessarily by interfering with the biosynthetic potential of the gland (Zhu and Noriega, 2016). The CA is innervated by axons of peptidergic neurosecretory cells as well as typical neurons. Previous in vitro studies have established that brain factors in mosquitoes modulate CA activity (Li et al., 2006; 2004). These regulatory peptides, termed allatotropins and allatostatins have stimulatory and inhibitory effects on CA activity, respectively. In A. aegypti, both AST-C and allatotropin have been identified in the brain (Li et al., 2004). AST-C, acts in a strong, rapid, and reversible manner. Inhibition of JH synthesis takes place when AST-C interferes with the citrate carrier (CIC) and prevents the transport of citrate across the mitochondrial membrane into the cytosol; blocking the production of Acetyl-CoA, a principal building block of JH (Nouzova et al., 2015). In various insects like D. melanogaster and the cockroach B. germanica, the insulin pathway has been shown to modulate JH synthesis (Maestro et al., 2009 (Belgacem and Martin, 2007). Likewise, in A. aegypti insulin signaling has shown to be a modulator of JH synthesis by the CA. In vitro studies have shown that insulin stimulates JH synthesis and that inhibition of the Insulin/TOR pathway downregulates transcript levels of several JH biosynthetic enzymes (Pérez-Hedo et al., 2013). Additionally, starvation results in a decrease in JH synthesis via a decrease in insulin signaling which can be reversed by insulin stimulation (Pérez-Hedo et al., 2014). Remarkably, starvation results in the upregulation of insulin
receptors which allows the gland to amplify insulin signaling at low concentrations, while maintaining the biosynthetic potential of the CA.

In A. aegypti mosquitoes, developmental regulators like ETH and 20E play roles in the regulation of JH synthesis by synchronizing CA activity with developmental events such as ecdysis (Areiza et al., 2015; 2014). For the newly eclosed female, reactivation of the gland is essential for the orchestration of ovarian reproductive maturation mediated by JH. In essence, in the last hours of the pupal stage these regulators direct the precise timing of CA reactivation which precede adult metamorphosis. Changes in enzymatic activity affect the rate of JH synthesis by controlling the progression of isoprenoids through the biosynthetic pathway. For pharate adult (late pupa) and newly eclosed females, enzymatic activity in the late steps of the JH biosynthetic pathway is critical for increases in JH synthesis (Rivera-Perez et al., 2014; Areiza et al., 2014). In the pupal stage ETH and 20E act as allatotropic regulators of JH synthesis, by increasing the enzymatic activity of JHAMT; which transforms farnesoic acid into methyl farnesoate in the CA (Figure 5). In contrast, for blood-fed mosquitoes it is the activity of farnesol dehydrogenase (FALDH), the enzyme that transforms FAL into FA, which is the rate-limiting step in JH synthesis (Rivera-Perez et al., 2013). The work of this dissertation suggests that 20E modulates CA reactivation of the blood-fed female in a similar fashion to the pupa, but instead of JHAMT, its target is FALDH.
Conserved among insect orders ETH is a small C-terminally amidated peptide, that is and is a major regulator of ecdysis, or molts, in insects (Adams et al., 2006; Zitnan et al., 2007). Ecdysis triggering hormone is synthesized and secreted into the hemolymph by specialized endocrine cells called Inka cells that are located along branch points of lateral epitracheal trunks (Adams et al., 2006; Predel et al., 2010). Work on Manduca sexta and Bombyx mori have shown high expression of ecdysis triggering hormone receptors (ETHRs) in the CA suggesting a role for ETH in the regulation of JH synthesis (Zitnan et al., 2007; Yamanaka et al., 2008). Likewise, the pupae of A. aegypti mosquitoes, express ETHRs in the CA-CC which rises in synchrony with 20E levels in the four hours prior to eclosion (Areiza et al., 2014). The preparatory phase of ecdysis is mediated by rising 20E levels that induce gene expression of ETH and their cognate receptors (Shlyueva et al., 2014; Zitnan and Adams, 2012; Adams et al., 2006; Zitnan et al., 2003, 2003, 1999). In vitro stimulation of the CA with ETH showed an increase in both JHAMT activity and JH synthesis which is attributed to its effect on the mobility of intracellular calcium stores. Here, inhibition of the IP$_3$-operated mobilization of endoplasmic reticulum Ca$^{2+}$ stores results in a decrease of the observed ETH dependent increases of the CA (Areiza et al., 2014). The RNAi mediated silencing of ETH receptors in pupa further corroborated the stimulatory effect of ETH on JH biosynthesis; resulting in a reversal of the physiological effect of ETH on JH synthesis (Areiza et al., 2014).

More recently, the allatotropic role of ETH has also been demonstrated in D. melanogaster where it has been shown to maintain JH levels that are critical in
ensuring reproductive success (Meiselman et al., 2017; Lee et al., 2017). Similarly, ETH induced calcium mobilization in the CA of Drosophila and knockdown of ETHRs in the CA led to reduced JH levels (Lee et al., 2017).

The ecdysteroid hormone, 20E is the key hormonal regulator of developmental transitions such as molting and metamorphosis in insects (Lafont, Rene, et al., 2003). Pulses of 20E ensure proper developmental timing of behavior and physiological changes in insects during molting (Ashburner et al., 1974; Cherbas et al., 1991; Riddiford, 1993; Cherbas et al., 2000). These pulses have been proposed to play a role in JH synthesis in the M. sexta, B. mori, A. aegypti, and Drosophila (Liu, et al., 2018; Areiza et al., 2015; Kaneko et al., 2011, 2013; Whisenton et al., 1985, 1987; Rankin et al, 1986; Gu and Chow, 1996). Recently, a study in D. melanogaster has revealed a mechanism of cross-talk between 20E and JH in the regulation of metamorphosis through regulation of each other’s biosynthesis (Liu, et al., 2018). And in the larvae of B. mori, 20E appears to modulate synthesis by controlling the expression of some of the JH biosynthetic enzymes (Hiruma and Kaneko, 2013). The work of this dissertation has shown that in A. aegypti, 20E is a developmental regulator of JH synthesis; which during metamorphosis also acts as a developmental signal that ensures proper reactivation of JH synthesis in the CA of the late pupa (Areiza et al., 2015).
2.2.3 Mechanism of Action of JH

Once JH is secreted from the CA, it is transported in the hemolymph by specialized high affinity binding proteins to act at distant peripheral sites. In addition to enhancing the solubility of JH in the hemolymph, these proteins referred to as juvenile hormone binding proteins (hJHBPs), protect JH from degradation by enzymes (Goodman and Cusson, 2012; Nijhout, 1994; Trowell, 1992). For many decades the molecular mode of action of JH remained a mystery as the search for the JH nuclear receptor proved elusive. However, work by Charles, Jindra, and colleagues on *Tribolium castaneum* offered the first solid evidence of Methoprene-tolerant (MET) protein as the primary JH receptor (Charles *et al*, 2011). Methoprene-tolerant protein is a member of the basic helix-loop-helix PAS (bHLH-PAS) family of transcription factors which usually form homo- or heterodimers (Ashok *et al*., 1998). When MET is present as a homodimer it is inactive. However, in the presence of JH, MET undergoes a conformational change which allows it to bind to another bHLH-PAS domain-containing steroid receptor coactivator to form the functional heterodimeric receptor complex. In *Aedes aegypti* it is the “Ftz-F1-interacting steroid receptor coactivator” (FISC); the ortholog in *Drosophila* is “Taiman” (Tai); and in *Tribolium castaneum*, “steroid receptor coactivator “(SRC) (Zou *et al*., 2013; Bai *et al*., 2000; Charles *et al*, 2011). Juvenile hormone signaling is transduced via its functional receptor (MET-FISC) which translocates into the nucleus and binds to regulatory elements of DNA thereby controlling gene expression. These regulatory regions are located in the promoters of JH responsive genes are
known as juvenile hormone response elements (JHRE) that contain E-box sequences (Figure 6).

2.2.4 Discovery of 20-hydroxyecdysone (20E)

The discovery of 20E as the key molting hormone in the 1950s was established in part by the pioneering work of Stephan Kopec on the gypsy moth *Lymantria dispar* (Kopec, 1917, 1922). In his early ligation experiments, he disrupted development of final instar larvae and discovered that the brain secreted a humoral factor that was initiating the molting process. He placed a ligature in middle of the body effectively blocking circulation from the brain to the other half of the body; the anterior portion of the larvae proceeded to the pupal stage while the posterior did not (Kopec, 1917). What he did not anticipate was that indeed his observations were keying in on the interplay of three different hormones during the molting process, which would later come to be known as the “classical scheme of insect endocrinology”. Namely, that the actions of prothoracicotropic hormone (PTTH), ecdysone (E), and JH result in molting and metamorphosis in insects. Work by Wigglesworth in 1934 on *Rhodnius prolixus* corroborated the endocrine role of the brain and named the factor originating from the brain “brain factor.” By 1935, German researcher Gottfried Fraenkel performed neck ligation experiments on blowfly larva, *Calliphora erythrocephala* which corroborated the existence of a hormone resulting in pupation (Fraenkel, 1935). These ligation experiments would become the major molting hormone assay and called the “Calliphora test.” This assay would go on to be used for the isolation of ecdysone (Butenandt and Karlson, 1954). In 1941, Soichi Fukuda
performed double ligation experiments on *B. mori* larvae which showed that the molting hormone originated from the prothorax and not the brain (Fukuda, 1940). A breakthrough in the discovery of ecdysone came in 1952 when Carroll Williams reconciled the discoveries by Kopec and Fukuda in the control of molting. Carroll used diapausing pupae of the American silkworm, *Hyalophora cecropia* to elucidate the role of the brain hormone and the molting hormone (Carroll, 1952). Through implantation studies, he established that the brain secreted a hormone (PTTH) which activates a pair of glands (PGs) in the prothorax to secrete the molting hormone. In 1954, Peter Karlson and Butenandt isolated and purified 25 mg of ecdysone crystals (α-ecdysone, E) from 500 kg of silk moth pupae (Butenandt and Karlson, 1954). As a result of the technical constraints of the time, the chemical structure could not be ascertained from such a small amount and it would take eleven years for it to be derived. The first glimpses that ecdysone regulates gene expression came in 1960 when Karlson and Clever showed that injection of 20E into the fourth instar larvae of the midge, *Chironomus tentans* was responsible for the puffing of the salivary gland polytene chromosomes (Clever and Karlson, 1960). These puffs were correctly interpreted as an increase in transcriptional activity induced by 20E (Jones and Ou, 2013). Finally, more than a decade since its isolation, the structure of ecdysone was elucidated using X-ray crystallography analysis (Hampshire and Horn, 1966; Hocks and Weichert, 1966; Huber and Hope 1965; Karlson et al., 1965). The biologically active metabolite, 20-hydroxyecdysone (20E) is the primary molting hormone in all arthropods and was initially isolated in crayfish
(Hampshire and Horn, 1966). The definitive identification of the PGs as the source of ecdysone was demonstrated in analysis of in vitro cultures of PGs derived from several insects including, *M. sexta, B. mori* (King et al., 1974; Chino et al., 1974). By 1975 the role of 20E in the control of reproduction was discovered when Hagedorn demonstrated that the ovaries of the mosquito *A. aegypti* are a source of ecdysone in the adult (Hagedorn, et al., 1975).

### 2.2.5 Ecdysone biosynthesis

The steroid hormone ecdysone (E) is a master regulator of insect developmental transitions. In arthropods, 20E is the key regulator of embryonic development, molting, metamorphosis, and reproduction. The site of synthesis for the ecdysteroid differs between the immature and adult stages of insects. For instance, during the larval stages of most insects, ecdysone biosynthesis takes place in the prothoracic glands. However, in the adult, the glands degrade, and the ovarian follicle cells become the source of ecdysone (Goltzené et al., 1974; Hagedorn, et al., 1975). *In vitro* studies in *A. aegypti*, have shown that larva and pupa synthesize ecdysteroids in thoracic and abdominal tissues (Jenkins et al., 1992). In *A. aegypti*, the prothoracic glands degenerate within 24 h after eclosion and the ovaries become the source of ecdysone (Raikhel, 2005; Hagedorn, et al., 1975). Once secreted, ecdysone is rapidly converted by a P450 monooxygenase into 20E, its biologically active form. The activation takes place in peripheral tissues like the fat body, midgut, and epidermis (Yamanaka et al., 2013; Riddiford, 2012; Nijhout, 1994).
Insects cannot synthesize cholesterol de novo and thus absorb sterols, the precursors of ecdysteroids, from dietary sources. The exact steps in the biosynthesis of 20E from cholesterol have eluded identification but there have been advances in the characterization of the terminal enzymatic steps of steroidogenesis (Figure 7). In the 1980s, a breakthrough in the identification of these terminal steps came from embryonic lethal phenotypes observed in D. melanogaster mutants by researchers Christiane Nüsslein-Volhard and Eric Wieschaus (Jürgens et al., 1984; Nüsslein-Volhard, et al. 1984; Wieschaus et al., 1984). Mutants displayed disrupted formation of embryonic cuticle because of low ecdysteroid titers (Chávez et al., 200). In the tradition of naming Drosophila genes after the related physical trait, they named the cuticle mutants, “disembodied,” “phantom,” “shadow,” “shade,” and “spook.” O’Connor, who contributed to identifying one of the enzymes (Dib) in the terminal steps would coin the term “Halloween genes” to classify the gene family. In total, the Halloween genes include, spook (Spo), spookier (Spok), phantom (Phm), disembodied (Dib), shadow (Sad), and shade (Shd) (Niwa & Niwa, 2014; Rewitz, et al., 2006; Gilbert, 2004.).

Halloween genes encode cytochrome P450 enzymes (CYP), which are involved in the biosynthesis of 20E. To date, the early steps in the pathway remain elusive and are collectively termed the “Black Box.” Steroid biosynthesis begins with the conversion of cholesterol into 7-dehydrocholesterol (7dC) by the (Rieske-domain protein) enzyme Neverland (Nvd) (Yoshiyama-Yanagawa et al.,
2011; Yoshiyama et al., 2006). Then by a series of yet unidentified enzymes, 7dC is converted into 3β,14α-dihydroxy-5β-cholest-7-en-6-one (5β-ketodiol). Thus far, four enzymes have been hypothesized to act in the conversion steps of the “Black Box”: CYP307A1/Spook (Spo), CYP307A2/Spookier (Spok), CYP6T3, and Non-molting glossy/Shroud (Nm-g/Sro) (Ou et al., 2011; Niwa et al., 2010; Ono et al., 2006; Namiki et al., 2005). In the terminal steps of 20E biosynthesis, 5β-ketodiol is sequentially hydroxylated by four P450 enzymes among them are, CYP306A1/Phantom (Phm), CYP302A1/Disembodied (Dib), CYP315A1/Shadow (Sad), and CYP314A1/Shade (Shd) (Warren et al., 2004; 2002; Petryk et al., 2003; Chávez et al., 2000). The last three steps in the biosynthesis of ecdysone take place in the PGs and are mediated by the enzymes, Phm, Dib, and Sad. In the final reaction of biosynthesis, ecdysone is released into circulation and converted in peripheral tissues into the active hormone 20E by the enzyme Shad.

### 2.2.6 Ecdysone mode of action

During metamorphosis, 20E triggers a transcriptional cascade of early and late genes which is referred to as the Ashburner model (Hill et al., 2013; Thummel, 2002). The Ashburner model was first proposed as a model for the hormonal regulation of puffing activity of polytene chromosomes in the salivary glands of Drosophila (Ashburner, et al., 1974; Ashburner and Richards, 1976). The effect of ecdysteroids on gene transcriptional activity was first demonstrated in the 1960s in studies on the puffing patterns of the giant polytene chromosomes of the midge, Chironomous tentans (Clever and Karlson, 1960). Clever and Karlson discovered that injection of ecdysteroids in third-instar larva
of *C. tentans* induced two sequences of puffs at specific loci of the chromosomes (Clever and Karlson, 1960). Moreover, they observed that the early puffs occurred within minutes after injection and did not require prior protein synthesis in contrast to the late puffs. Michael Ashburner and colleagues would go on to define the molecular mode of action of 20E. His model would provide a conceptual framework for the orchestration of transcriptional responses that ultimately leads to molting, metamorphosis, and reproductive maturation in insects. In his studies on larvae of *D. melanogaster*, Ashburner demonstrated that if translation was inhibited by cycloheximide treatments it interrupted the appearance of the late puffs and prevented regression of early puffs (Ashburner *et al.*, 1974). Thus, he posited that early puffs were a result of a direct transcriptional response to 20E; and the proteins encoded by these genes are responsible for regulation of later puffs. Additionally, as these proteins become more abundant they feedback to repress their own expression.

According to this model, 20E bound to its receptor directly activates expression of a small number of early or primary-response genes. In turn, the protein products of these genes are nuclear receptors with the dual role of activating expression of larger group of late genes, while repressing their own expression. Unique to metazoans, nuclear receptors are ligand-activated transcription factors that regulate gene expression (Bonneton and Laudet, 2012). They are activated by lipophilic ligands among them steroid hormones, thyroid hormones, and various other lipid soluble signals. In *Aedes aegypti*, 20E induces
the expression of several transcription factors that are also involved in vitellogenesis including *E74, E75, Broad-Complex (BR-C)*, and AHR3 (Chen *et al.*, 2004; Sun *et al.*, 2002; Kapitskaya *et al.*, 2000; Pierceall *et al.*, 1999).

The functional ecdysteroid receptor, is a heterodimer comprised of two nuclear receptors, ecdysone receptor (EcR) and ultraspiracle (USP), which is a homolog of the vertebrate retinoid X receptor (RXR) (EcR; Koelle *et al.*, 1991; USP; Oro *et al.*, 1990; Shea *et al.*, 1990; Henrich *et al.*, 1990). Both EcR and USP are members of the nuclear receptor superfamily and share basic structure organization (Riddiford *et al.*, 2000). The common structural organization of the receptor includes the N-terminal region A/B domain (transactivation), C domain (DNA binding domain), D region (hinge), E domain (ligand binding domain), and the C-terminus F domain which is found in all insect EcRs but not in any known USP (Figure 8) (Pawlak *et al.*, 2012; Bonneton and Laudet, 2012). The A/B domains are highly variable and are involved in transcriptional regulation, they interact with the transcriptional machinery of cells. In contrast, the C domain or the DNA-binding domain (DBD) is relatively well conserved among insects and arthropods and is also involved in the dimerization of nuclear receptors (Robinson-Rechavi *et al.*, 2003). The main function of the C domain is sequence recognition at DNA regulatory sites in the genome (Riddiford *et al.*, 2000). The D domain or hinge region acts as a flexible linker between the C and E domains. The E domain, or ligand binding domain (LBD), is the largest and interacts with
Some nuclear receptors have an F domain at the C-terminus, its function is not fully understood.

Upon 20E binding the heterodimer complex (EcR/USP), 20E translocates into the nucleus where the receptor complex controls target gene transcription (Figure 9). Here it directly activates the expression of 20E response genes by recognizing specific DNA sequences present in promoter regions known as ecdysone response elements (EcREs) (Margam et al., 2005; Hall and Thummel, 1998; Cherbas and Cherbas, 1996). In insects, there are various isoforms of EcR and USP and their differential expression and different heterodimer isoform combinations provide versatility to 20E mediated responses. For example, *Aedes aegypti* there are two isoforms for EcR (AaEcRA/B) and USP (AaUSPA/B) receptors and both are expressed in the CA before and after adult emergence (Wang et al., 2002; Wang et al., 2000; Kapitskaya et al., 1996). Recently, it has been suggested that in insects and crustaceans there might be alternative modes of action for ecdysteroids that might involve other signaling elements such as membrane-mediated mechanisms. Increasing evidence suggest that 20E can also mediate their effects in a non-genomic manner via the activation of GPCRs (Jing et al., 2015; Srivastava et al., 2004). Work in *D. melanogaster* has identified a GPCR that mediates 20E responses (Srivastava et al., 2004). This novel GPCR was called, *Drosophila melanogaster* dopamine/ecdysteroid receptor (*DmDopEcR*), with structure homology to the vertebrate “Y-adrenergic receptors”. As the name of the receptor implies, this GPCR can bind both
dopamine and 20E and modulate intracellular signaling cascades involving the activation of CaMKII (Lark et al., 2017; Jing et al., 2015).

2.3 Juvenile Hormone and 20-hydroxyecdysone endocrine partners in regulation of development and reproduction in Aedes aegypti

Juvenile hormone and 20E are two pleiotropic hormones that work in concert to control critical physiological events throughout ontogeny of insects. Namely these lipophilic hormones orchestrate molting and metamorphosis in immature insects and reproductive maturation in the adult (Flatt et al., 2005; Hartfelder, 2000; Riddiford, 1994). In the immature insect, a rise in 20E titers initiates molts and metamorphosis (Cherbas et al., 2000; Riddiford, 1980). While, the developmental outcome of the molt is determined by the titer of JH in the hemolymph. Thus, a balance between these two major hormones dictate developmental transitions in insects. After metamorphosis, the two endocrine partners are responsible for mediating ovarian maturation as they coordinate events in the vitellogenic cycle. The following sections aim to describe in more detail the roles that JH and 20E play in development and reproduction.

2.3.1 Endocrine Control of Molting and Metamorphosis by JH and 20E

In insects, developmental transitions such as molting and metamorphosis are under strict endocrine control, a fact especially true for holometabolous insects like mosquitoes. The life cycle of Aedes aegypti is composed of four stages 1) embryonic (egg), 2) larval, 3) pupal, and 4) adult (Figure 10). Two hormones that work in conjunction to direct these developmental transitions are
the sesquiterpenoid JH and the ecdysteroid 20-hydroxyecdysone (20E). Pulses of 20E with unique amplitudes and duration induce transcriptional cascades resulting in larval growth and the reorganization of body plan that typifies metamorphosis (Ou and King-Jones, 2013; Riddiford, 1994). The molting process begins with the secretion of the neuropeptide hormone PTTH from neurosecretory cells in the brain (Figure 11). In turn, PTTH stimulates the biosynthesis and secretion of ecdysone by the prothoracic glands. Ecdysone is the pro-hormone of the biologically active form, 20-hydroxyecdysone (20E). Once released, ecdysone is taken up at target tissues where it is converted to the active form, 20E. Known as the “molting hormone,” 20E binds its nuclear hormone receptor and initiates molts and metamorphosis through a transcriptional cascade that results in larval growth and the reorganization of body plan that typifies metamorphosis. The immediate effect of 20E on epidermal tissues include apolysis (separation of the old cuticle), cell division, digestion of old cuticle, and secretion of the new cuticle (Zitnan and Adams, 2012; Nijhout, 1994). In addition, 20E also mediates ecdysis, by regulating the synthesis and secretion of ETH along with expression of its cognate receptors (Dai and Adams, 2009; Kingan and Adams, 2000). Ecdysis is the shedding of the old exoskeleton at the end of each molt and is part of an innate behavioral sequence of stereotypical movements. The ecdysis sequence is comprised of rhythmic contractions and relaxation that loosen the old cuticle and peristaltic movements that aid its shedding (Zitnan et al., 2003; Margam et al., 2006; Adams et al., 2006).
The action of JH in the immature insect is mainly antimetamorphic as it preserves the “status quo” of the body plan during the molt. During larval development, JH in the presence of 20E ensures that juvenile characteristics are retained, and the molts are from one smaller larval instar to a successively larger larval instar. Alternatively, a rise of 20E in the absence of JH allows for the metamorphic progression seen in larval-pupal or pupa-adult molts. Holometabolous insects like A. aegypti mosquitoes, have small pockets of cells that invaginate from the epidermis at various locations in the early stages of embryonic and larval development. These cells form the imaginal discs and they remain undifferentiated growing slowly during the larval stages. Imaginal discs are the are tissue-specific progenitors that give rise to the adult body structures like compound eyes, limbs and genitalia (Aldaz et al., 2010; Nijhout, 1994). Juvenile hormone suppresses the precocious growth and differentiation of primordia and imaginal discs (Jindra et al., 2013; Truman et al., 2006). After hatching, Aedes undergoes four larval instar molts, in the last larval stadium a rise in 20E coupled with the absence of JH signals a cessation of feeding and onset of pupation (Margam et al., 2006). The pupal stage in A. aegypti mosquitoes lasts approximately 50 hours. During this stage a pharate adult, that is, an adult encased in the pupal cuticle, develops until the metamorphic molt takes place (eclosion). Surges of 20E allow the mosquito to undergo major developmental restructuring as it becomes the pharate adult. A large pulse of 20E early in the pupal stage initiates the adult transformation and apolysis (Margam et al., 2006). While smaller pulses, hours prior to eclosion provides
temporal cues for the execution of ecdysis and ensures proper reactivation of JH synthesis in the mosquito pupae (Areiza et al., 2015; Margam et al., 2006).

2.3.2 Hormonal control of reproduction in *Aedes aegypti* by JH and 20E

Juvenile hormone and 20E, the principal hormones that controlled developmental processes in immature insects, go on in the adult insect to govern reproduction. In the female mosquito, hormonal control of reproduction by these two master regulatory hormones has been extensively studied. For anautogenous mosquitoes like *A. aegypti*, a blood meal is required to produce a clutch of eggs (Nijhout, 1994). Juvenile hormone and 20E regulate the progression of the gonotrophic cycle through a biphasic cyclic fluctuation of their levels that is mediated by nutritional status (Briegal, 1990; Noriega, 2004). Oogenesis can be divided into three discrete periods, 1) previtellogenesis, 2) ovarian resting stage, and 3) vitellogenesis (Figure 12) (Klowden, 1997). Together, JH and 20E orchestrate aspects of reproduction which include, previtellogenic development, ovarian maturation, yolk protein synthesis, and choriogenesis. The first two periods of the vitellogenic cycle are controlled by JH; while vitellogenesis and the subsequent completion of egg development is mediated by 20E. The site of synthesis for JH remains the same, however, in mosquitoes the PGs degenerate after metamorphosis and the ovaries becomes the source of ecdysone (Hagedorn et al., 1975; 1979)

The ovaries of *A. aegypti* mosquitoes contain approximately 50 to 150 ovarioles. In newly emerged mosquitoes, an ovariole consists of an anterior
germarium, attached to a secondary follicle, and primary immature follicle. In the first three days post eclosion, female mosquitoes feed on a nectar diet and undergo a period of maturation that is orchestrated by a rise in JH titers. At emergence, they synthesize low amounts of JH which steadily rises to a peak at 12 h (90 fmol/CA/hour) (Rivera-Perez, et al., 2014). The rise of JH is an essential component of the previtellogenic preparatory period and mediates physiological adaptations for blood feeding. During this developmental period, JH prepares the mosquito for blood digestion by regulating transcription of the early trypsin gene in the midgut (Noriega et al., 1997). Additionally, JH exposure leads to competence of the fat body, the equivalent to the vertebrate liver, to synthesize massive amounts of yolk protein precursors upon a rise of 20E titers (Flanagan and Hagedorn, 1977; Adams, 2005; Riddiford 2012). Likewise, JH regulates reproductive output by maturing the ovaries and inducing their competence to respond to hormones and uptake vitellogenin (Vg), a major yolk protein, into developing oocytes (Raikhel and Lea, 1985; Shapiro and Hagedorn, 1982; Gawdz and Spielman, 1973). At eclosion, the primary follicles of newly emerged females are undifferentiated and are approximately 40 – 50 µm long (Koeppe et al., 1985; Gawdz and Spielman, 1973). During the previtellogenic period, JH acts on the ovaries by maturing the primary follicles, as they double in size to 100 µm. At the end of 48 – 72 hours, the now mature primary follicles enter a state of reproductive arrest, or ovarian resting stage, in which development ceases until a blood meal is ingested (Hagedorn et al., 1977). Furthermore, JH mediates the previtellogenic resorption of follicles in response to
nutritional constraint thereby affecting the number of eggs produced (Cliffton et al., 2011; 2012). After a blood meal, the state of arrest comes to an end and vitellogenesis commences as the primary follicles develop synchronously (Raikhel and Lea, 1990).

Blood ingestion initiates a cascade of vitellogenic events in which 20E acts on several tissues resulting in the deposition of yolk proteins into developing oocytes and their subsequent oviposition. Immediately following a blood meal, abdominal stretch and nutritional signals prompt neurosecretory cells in the brain to synthesize two types of neuropeptide hormones; namely, ovarian ecdysteroidogenic hormone (OEH) and insulin-like peptides insulin-like peptides (ILPs), from medial neurosecretory cells in the brain (Riehle and Brown, 1999; Klowden, 1987). In adults, ecdysone synthesis is stimulated by OEH, which substitutes the role of PTTH (Figure 11). OEH, also known as the ovarian ecdysteroidogenic hormone (EDNH) is secreted by the CC and stimulates the ovarian follicle cells to produce E which will be converted by a P450 monooxygenase into 20E, its biologically active form in the fat body. (Hagedorn et al., 1975). After a blood meal, ecdysteroid titers rise and peak at 18–24 h post blood meal (PBM) which decline to basal levels by 36 hours PBM. In contrast, blood ingestion causes a dramatic drop in JH synthesis during the first three hours, and levels reach their lowest point 24 h later (Rivera-Perez et al., 2014). During vitellogenesis, 20E acts on the fat body, to synthesize yolk protein precursors (YPPs). For developing oocytes, the major YPP is vitellogenin (Vg).
Vitellogenin is secreted into the hemolymph and is sequestered into the developing oocyte via-receptor mediated endocytosis. After the incorporation of Vg, they are processed further into the essential nutrients that are essential for embryonic development. As vitellogenesis is taking place in the primary follicle, 20E will stimulate the separation of the secondary follicle in each ovariole. Forty-eight hours post blood meal, JH levels begin to rise again and return to pre-blood meal levels on the third day (Li et al., 2003; Shapiro et al., 1986). The 48h PBM rise in JH mediates the previtellogenic growth of the secondary follicle, preparing the stage for the second gonotrophic cycle (Shapiro et al., 1986). Declining levels of 20E mark the cessation of vitellogenesis as the fat body stops expressing Vg and the final steps in choriogenesis take place preparing the egg for oviposition at approximately 72 h PBM (Hagedorn, 1985).

2.4 Concluding remarks

The foundation for modern insect endocrinology is indebted to the pioneering work of researchers in the 19th and early 20th century. Their findings ultimately led to the discovery of endocrine regulation of molting and metamorphosis by JH and 20E. These were followed by groundbreaking discoveries regarding their roles in orchestrating reproductive maturation and their mode of action. Considering the important developmental and gonotrophic roles of JH, it is not surprising that biosynthesis of the hormone is under strict regulation. In the life history of A. aegypti mosquitoes, JH biosynthesis is a dynamic process in which the glands alternate between four distinct physiological states, inactive, active, modulated, and suppressed (Zhu and Noriega, 2016;
Rivera-Perez et al., 2014). For instance, as the antimitamorphic role of JH comes to an end the CA is seemingly inactive in the pupal stage, however the CA must become active to ensure previtellogenic development in the newly eclosed female. In *Aedes aegypti*, rising 20E titers in the late pupal stage temporally correlate with JH levels, suggesting a role for the ecdysteroid in the activation of the CA in the late pupa (Areiza et al., 2015; Margram et al., 2006). In the blood fed female, the CA must also alternate between physiological stages of suppression and reactivation to ensure the proper completion of vitellogenesis and the initiation of the second gonotrophic cycle. The aim of the dissertation is to elucidate the role of 20E in regulation of JH biosynthesis as it transitions through some of these physiological states. The completion of these aims has demonstrated that, in the last hours prior to eclosion, developmental factors like 20E mediate the reactivation of the CA (Areiza et al., 2016; 2015). In addition, we have found that 20E acts a trophic factor on the CA of the blood fed female which ensures progression of the secondary gonotrophic cycle.
Figure 2. Chemical structures of examples of naturally occurring juvenile hormone (JH) homologues and three JH analogs (agonists).
Figure 3. The Juvenile Hormone biosynthetic pathway. The pathway is divided into two steps: early and late steps which involve 13 sequential enzymatic reactions. The early steps go by the way of the mevalonate pathway; the later steps are unique to JH-biosynthesis.
Figure 4. Proposed model for the control of JH synthesis by allatoregulators. Ecdysis-triggering hormone (ETH), 20-hydroxyecdysone (20E), allatostatin-C (AST-C), insulin (INS), and allatotropin (AT).
Figure 5. Flux of precursors in the JH biosynthetic pathway of pupae. Ovals represent the precursor pools: Farnesal (FAL), Farnesoic Acid (FA), Methyl Farnesoate (MF). The proposed modulators, ecdysis-triggering hormone (ETH) and 20-Hydroxyecdysone (20E) are in diamonds. Modulators affect enzymatic activity of Juvenile hormone acid methyl transferase (JHAMT) seen in red.
Figure 6. JH Mode of signaling. JH signaling is transduced via its functional receptor (MET-FISC) which translocates into the nucleus and binds to regulatory elements of DNA. In the presence of JH, MET undergoes a conformational change which allows it to bind to another bHLH-PAS domain-protein, the steroid receptor coactivator, Ftz-F1-interacting steroid receptor coactivator (FISC). JH and its functional receptor bind directly to the promoters of JH responsive genes termed juvenile hormone response elements (JHRE) that contain E-box sequences.
Figure 7. Ecdysteroid biosynthesis pathway and ecdysteroidogenic enzymes in insects leading to 20E. Arrows between 7dC and the 5β-ketodiol indicate an uncharacterized series of oxidations called the ‘Black Box’ reaction that may involve P450 enzymes. In the terminal steps, 5β-ketodiol is sequentially hydroxylated by four P450 enzyme: Phantom (Phm), Disembodied (Dib), Shadow (Sad), and Shade (Shd).
Figure 8. Generalized structure of the nuclear receptor superfamily. The ecdysone receptor (EcR) and ultraspiracle (USP) share a common structural organization. Constructed from five domains (A/B, C, D, E, F), the receptor includes a N-terminal region A/B domain (transactivation), C domain is the DNA binding domain (DBD), D region (hinge), E domain is the ligand binding domain (LBD), and a C-terminus F domain.
Figure 9. 20E Mode of signaling. 20E signaling is transduced via its functional receptor (EcR/USP) which translocates into the nucleus and binds to regulatory elements of DNA. 20E activates the expression of target genes by recognizing specific DNA sequences present in promoter regions known as ecdysone response elements (EcREs).
Life cycle of *Aedes aegypti*

*Figure 10. Diagram depicting the life cycle of Aedes aegypti.* Female mosquitoes lay their eggs, eggs hatch into larva when water inundates the eggs. After hatching larva undergo four larval instar molts. At the end of the fourth instar, larva pupate, molt in to a pupa. Approximately 48 hours later, pupa emerge into the adult mosquito.
Figure 11. Hormonal cascade leading to 20E synthesis in immature and adult insects. In immature insects, neurosecretory cells in the brain secrete the neuropeptide hormone prothoracicotropic hormone (PTTH) which stimulates the biosynthesis and secretion of ecdysone (E) by the prothoracic glands (PGs). In adults, after a blood meal the brain releases ovarian ecdysteroidogenic hormone (OEH) which the is secreted by the CC. In both cases, E is converted to its biologically active form, 20E by peripheral tissues.
Figure 12. Representation of hormonal control of the vitellogenic cycle in *Aedes aegypti*. Three discrete periods of oogenesis are depicted in green ovals: 1) previtellogenesis, 2) ovarian resting stage, and 3) vitellogenesis. Previtellogenesis and ovarian resting stage are controlled by JH; while vitellogenesis and the subsequent completion of egg development is mediated by 20E. A blood meal stimulates the synthesis of 20E which initiates vitellogenesis and the separation of the secondary follicle. Reactivation of the CA begins the second gonotrophic cycle by JH.
References


Chapter 3: Advances in mosquito transgenesis

“Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.”

Sydney Brenner

3.1 Introduction

In 1982, Ruben and Spradling were the first researchers to demonstrate germline transformation of an insect. Working with *D. melanogaster*, they were able to introduce exogenous DNA sequences using a P transposable element (Ruben and Spradling, 1982). After the discovery of P element mediated transformation in *D. melanogaster*, there was hope that this system could be harnessed for transgene integration into other insects. However, traditional drosophilid methods of germ-line transformation through the P vector failed as they were genus specific. Mosquito transgenesis as a molecular approach was elusive for some time, but by 1998 other transposon-based vectors proved to be successful in transformation of non-drosophilid insects including *A. aegypti* and other mosquitoes (Coates *et al.*, 1998; Jasinskiene *et al.*, 1998). The four class II transposable elements used for transformation in mosquitoes are *piggyBac*, *Hermes*, *Mos1* (mariner), and *Minos* (Coates *et al.*, 1998; Jasinskiene *et al.*, 1998; Catteruccia *et al.*, 2000; Lobo *et al.*, 2002).

Notwithstanding the advances made by transposon vector systems, there are some limitations which include the potential risk of transgenic instability (Adelman *et al.*, 2002; Fraser, 2013). The use of site-specific recombination systems attempted to mitigate these effects, but they require the integration of a “docking site” into the genome (Schetelig *et al.*, 2009; 2011). Until recently,
transgene integration had consisted of transposable elements and site-specific recombination systems. However, recently established advances in genome editing that use custom-engineered nucleases have enabled the precise manipulation of specific genomic sequences. Generally, three types of sequence-specific nucleases are used, namely, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat-associated endonucleases (CRISPR/Cas) (Figure 1). The chapter reviews recent advances in transgene integration techniques, with an emphasis on Aedes aegypti mosquitoes.

3.2 Transposon mediated gene transfer

In transposon mediated gene transfer, the most common form of germ-line transformation in insects, in which a vector and a helper plasmid are co-injected prior to pole cell formation into preblastoderm embryos (Handler, 2001). P elements are class II transposable elements – mobile genetic elements that move by a cut-and paste-mechanism – that excise from the donor site and are reinserted elsewhere in the genome (Finnegan, 1992; Beal and Rio, 1997). These elements are small (1-3 kb) and are characterized by having a central transposase gene which is flanked by inverted terminal repeats (ITRs). Usually the vectors are non-autonomous; the transposase gene is removed to prevent self-mobilization (James et al., 2002). As such, transposon-mediated systems were designed to work as a binary system composed of two separate plasmids in which one plasmid, the vector or donor, carries the transgene and is flanked by ITRs (Lobo et al., 2006). The second plasmid, the helper, provides the
transposase in \textit{trans} and is under the control of an inducible promoter. Since the helper plasmid lacks the terminal inverted repeats, it will not integrate into the genome.

The first germline transformation was achieved in \textit{Drosophila melanogaster} when exogenous DNA sequences containing the wild-type \textit{rosy} gene (\textit{rosy+}) was introduced by a \textit{P} transposable element. Transformation of \textit{rosy+} was verified using an eye color marker. Since the injected embryos were \textit{rosy-} mutants, transformants would exhibit wild-type eye color which was stably inherited in subsequent generations (Ruben and Spradling, 1982). The framework of the binary vector/helper transformation scheme would go on to be used in other transposon-based systems. While \textit{P} transformation proved to be elusive in \textit{Aedes} mosquitoes it did serve as a model for future transposon mediated transformations. Transformation in mosquitoes finally became a reality using another set of class II transposable elements, \textit{Hermes}, \textit{Mos1} (\textit{mariner}), \textit{Minos}, and \textit{piggyBac} (Sarkar et al., 1997; Coates et al., 1998; Lobo et al., 1999).

\textbf{3.2.2 Hermes}

In 1997 Sarkar and colleagues showed that \textit{Hermes}, a short-inverted repeat type transposable element from the housefly \textit{Musca domestica}, was able to transpose into embryos of \textit{A. aegypti} mosquitoes (Warren et al., 1994; Sarkar et al., 1997). The application of Sarkar and colleagues allowed the first successful germline transformation of \textit{A. aegypti} using a modified \textit{Hermes} transposon to introduce the \textit{melanogaster cinnabar} (\textit{cn}) gene to embryos of a kynurenine hydroxylase-deficient (\textit{kh}w), white-eyed recipient strain (Jasinskie
et al., 1998). However, there have been some atypical mechanisms of movement. For instance, a portion of the donor plasmid sequences have incorporated into the mosquito genome (Adelman et al., 2002; Moreira et al., 2003; Fraser, 2012).

### 3.2.3 Minos

Minos is a transposable element isolated from *Drosophila hydei* and is part of the mariner family, along with Mos1 (Franz et al., 1994). The first transposon mediated transformation in a non-drosophilid was performed by a Minos element on the Mediterranean fruit fly, *Ceratitis capitata* (Loukeris et al., 1995). The Minos element is widely used in transgenesis of insects (Uchino et al., 2007; Chu et al., 2017), including *Anopheles stephensi* mosquitoes (Catteruccia et al., 2000).

### 3.2.4 Mos1

The Mos1 mariner element was isolated from *Drosophila mauritiana* where it was associated with an unstable white mutation of the white-peach allele (wpch) (Jacobson et al., 1986). The functional Mos1 element consists of 1286-bp (transposase) and a pair of 28-bp TIRs (Jacobson et al., 1986). Putative mariner elements have been found in various insect orders (Robertson, 1993; Handler, 2001). Mos1 can transform a wide variety of insect species like *A. aegypti* (Coates et al., 1998), *D. melanogaster* (Garza et al., 1991; Lidholm et al., 1993), and cultured cells of *B. mori* (Wang et al., 2000). Remarkably, Mos1 has also been found to be active in the protozoan *Leishmania major* (Gueiros-Filho and
Beverley, 1997) as well as vertebrates like zebrafish and the chicken (Fadool et al., 1998; Sherman et al., 1998).

In *A. aegypti* mosquitoes, Mos1 mediated transformation has made possible a number of experimental approaches critical in this vector species (Travanty et al., 2004; Mathur et al., 2010). The first demonstration that Mos1 could transform the yellow fever mosquito was done using the *D. melanogaster cinnabar (cn+)* gene as a transformation marker for injected embryos of the *khw*, white-eyed recipient strain (Coates et al., 1998). While it has been shown that transposition follows the typical pattern of cut and paste, there have been instances of integration resembling that of the *Hermes* element, namely the integration of flanking sequences (O’Brochta et al., 2003).

### 3.2.5 PiggyBac

The *piggyBac* element (first known as IFP2) was originally isolated from the cabbage looper moth, *Trichoplusia ni* (Fraser et al., 1983). The element is part of a family of TTAA-specific elements and is 2.5kb in length with a pair of 13-bp TIRs (Fraser, 2013). The first germline-transformation in insects using *piggyBac* was accomplished in *Ceratitis capitata*, using the white-eye (*we*) marking system (Handler et al., 1998). Shortly after, the first *piggyBac* mediated transformations in *A. aegypti* mosquitoes were established (Lobo et al., 1999; Kokoza et al., 2001; Lobo et al., 2002). Lobo and colleagues demonstrated that *piggyBac* could transpose in the embryos of *D. melanogaster*, *A. aegypti*, and *T. ni* (Lobo et al., 1999). This would be followed by transformations in *A. aegypti* using the genetic markers cinnabar (*khw*) and 3xP3-EGFP to compliment white-
eye phenotype \((kh^w)\) strains (Kokoza et al., 2001; Lobo et al., 2002). Transpositions mediated by piggyBac follow the typical pattern of cut and paste and have been shown to mediate germline transformation in numerous insects including \(D.\ melanogaster\), \(B.\ mori\), and \(Tribolium\ castaneum\) (Lorenzen et al., 2003; Tamura et al., 2000; Handler and Harrell, 1999). Moreover, it has been extensively used in transformations of the \(Anopheles\) mosquitoes, important malaria vectors, including \(A.\ stephensi\) (Nolan et al., 2002), \(A.\ albimanus\) (Perera et al., 2002), and \(A.\ gambiae\) (Grossman et al., 2001).

### 3.3 Site-specific recombinases

One of the biggest challenges with transposon-mediated transformation is the potential target for positional effect. For instance, transposon-mediated insertions can result in expression variability because of the neighboring \(cis\)-regulatory DNA elements or even the local chromatin arrangement at sites of insertion. Site-specific recombinases offer an alternative to transposon mediated gene transfer. They have several advantages over transposons, including more reproducible insertion, higher integration frequency, and higher carrying capacity (Nimmo et al., 2006). Additionally, integration can be unidirectional which limits the loss of transgenes due to remobilization of homologous elements (Fraser, 2013). A caveat to their use is that natural insertion sites are not common and hence require prior insertion of a canonical integration site, or “docking site,” into chromosomes via transposon mediated transgenesis (Schetelig et al., 2009; 2011; Wimmer, 2005). Recombinases are a targeting system which allows for integrations to be limited to a defined target site, thus limiting unwanted positional
effects. The action of site-specific recombinases is mediated by a crossover event that involves the cleavage at the recognition site and reunion, or integration between sites. Site specific systems are thus composed of a transgene-bearing plasmid carrying the \textit{attB} site and a second donor plasmid containing the \textit{attP} which serves as the docking site for any incoming \textit{attB}-containing plasmid.

Recombinases are divided into two essential groups, the tyrosine and serine recombinases. In \textit{A. aegypti}, the serine integrase \textit{φC31} has been effectively used in genetic transformation (Chompoosri \textit{et al}., 2009; Nimmo \textit{et al}., 2006). The \textit{φC31} integrase is derived from bacteriophage \textit{φC31} and its recognition sites where recombination takes place between the \textit{attB} and \textit{attP} attachment sites. Two of the main advantages of the \textit{φC31} system are that it does not require co-factors and it has a large vectorial carrying capacity (Fraser, 2013; Pondeville \textit{et al}., 2014). The \textit{φC31} system has been implemented in other mosquito species such as \textit{A. albopictus}, \textit{An. Stephensi}, and \textit{An. gambiae} (Meredith \textit{et al}., 2011; Amenyia \textit{et al}., 2010; Labbé \textit{et al}., 2010).

3.4 Novel genetic approaches to genome editing: Zinc finger nucleases, transcription activator like effector nucleases, and CRISPR

More recent advances in mosquito transgenesis have been seen the advent of genome editing which allows for targeted genetic integration by using DNA nucleases. Three examples of this editing approach used in mosquitoes are: zinc-finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs), and the more recently developed CRISPR/Cas9 system (Aryan \textit{et al}., 2014; DeGennaro \textit{et al}., 2013; Kistler \textit{et al}., 2015). Common to all, the nuclease
creates double stranded breaks (DBS) in the target DNA which are then repaired by the DNA repair machinery either through non-homologous end joining (NHEJ) or homology directed repair (HDR). Here, in the presence of a homologous donor, DNA integration can take place via homologous recombination. The following sections give a brief look at each of these approaches.

3.4.1 Zinc-finger nuclease (ZFNs)

Zinc-finger nucleases (ZFNs) were the first genome editing approach to use engineered nucleases (Kim et al., 1996). Engineered nucleases, like ZFNs are modular proteins that are comprised of a non-specific DNA cleaving nuclease module that is fused to a sequence specific DNA binding domain (Gaj et al., 2013). The Fok I nuclease is a type IIS restriction endonuclease that has distinct DNA binding and DNA cleavage domains; the cleavage domains only become active if dimerized (Urnov et al., 2010; Carroll, 2011). The DNA binding domain is comprised of arrangement of zinc finger binding domains which recognize the target DNA sequence (Joung and Sander, 2013). Each single zinc-finger motif consists of approximately 30 amino acids in a conserved ββα structure which is stabilized by a zinc ion (Urnov et al., 2010; Miller et al, 1985). These zinc finger domains can be assembled using different combinations allowing for unique genome targets, making them an attractive tool for genome editing. For ZFNs to induce cleavage, two independent binding events must take place, where each zinc finger subunit binds the DNA target site, usually in a tail-to-tail conformation with appropriate spacing to facilitate the dimer formation. Upon dimerization, the DNA cleavage domains become enzymatically active and the target DNA
sequence is cut creating a fragment that is released. ZFNs have been successfully used to create targeted mutagenesis in *A. aegypti mosquitoes* (DeGennaro *et al.*, 2013; Liesch *et al.*, 2013; McMeniman *et al.*, 2014). DegGennaro and colleagues were the first to use the application of ZFNs in *A. aegypti* mosquitoes. Researchers injected mosquito embryos with ZFNs targeting Orco, part of the functional odorant receptor that forms a heterodimer with a variable subunit. Disruption in the expression of orco, allowed them to study the effect of loss-of-function of the olfactory receptor coreceptor (*orco*) gene in host selection and sensitivity to the chemical repellent DEET (DeGennaro *et al.*, 2013). Targeted mutagenesis by ZFNs has also been effectively used in other insects such as *D. melanogaster* and *B. mori* (Beumer *et al.*, 2006; Takasu *et al.*, 2010). However, there are some disadvantages with using ZFNs that have limited their use, such as their cost and time-consuming nature. For instance, off-target binding and consequent cleavage resulting in undesired modifications is a concern. These issues can be mitigated by sequencing the entire genome in order to control sequence specificity and also by testing more than one ZFN (Costa *et al.*, 2004).

3.4.2 Transcription activator-like effector nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) are a class of engineered nucleases which are modular proteins comprised of a *Fok I* nuclease domain and a DNA binding domain. This DNA binding domain is derived from highly conserved repeats of transcription activator-like effectors proteins (TALENs) that are produced by some plant pathogenic bacteria (*Xanthomonas*).
to alter gene transcription and regulate genes that promote infection in their host (Bogdanove et al., 2010; Bogdanove, and Voytas; 2011). Following the lead of work in ZFNs, TALENS were fused with the catalytic domain of Fok I and tested in a yeast assay in which LacZ activity serves as an indicator of DNA cleavage (Christian et al., 2010). Their mode of action is similar to ZFNs, where TALENs work as dimers forming a functional Fok I dimer that cleaves their target DNA. This DBS break in the absence of a donor plasmid results in error-prone NHEJ and consequently yields small deletions. Whereas, if a donor plasmid is present, the DBS break can be repaired through HDR and be used to introduce a desired sequence into the DNA genome (Joung and Sander, 2013). Work in the silk worm, B. mori, has demonstrated the use of TALENS in inducing targeted DNA mutations (Sajwan et al., 2013). In Sajwan’s study they generated three TALENs against the target sites of the BmBLOS2 genomic locus. BmBLOS2 is a marker gene of epidermal color which had been targeted in previous work by another engineered endonuclease, ZFN (Takasu et al., 2010). To verify the success of their three engineered TALENS they conducted a yeast reporter assay. Here, activity of α-galactosidase serves as an indicator of nuclease function. They found that indeed all three TALENs had activity and then proceeded to inject 80 preblastoderm stage embryos with each pair of TALENs. They were able to induce mutations with all three TALENS in germline cells of injected animals, with a higher success rate than the ZFN method.
3.4.3 CRISPR-Cas9

Lastly, the recent advent of the CRISPR/Cas9 system of genome editing has revolutionized the way of studying the function of genes. Like RNAi, the system was adapted from an endogenous system of gene expression control. The clustered, regularly interspaced, short palindromic repeat (CRISPR)/ and CRISPR associated (Cas) proteins were discovered to be part of an adaptive immune response in bacteria and archaea (Jansen et al., 2002; Bolotin et al., 2005; Doudna and Charpentier, 2014). As with RNAi, the CRISPR/Cas9 system uses small RNAs (~18-20 bp) to target specific nucleic acid sequences, but unlike RNAi, it base pairs with DNA target sequences (Figure 3). In particular, the CRISPR-Cas9 system is designed using the type II CRISPR-system that uses an endonuclease (Cas9), derived from *Streptococcus pyogenes* that is guided by a guide RNA (sgRNA) to locate specific locations of invading nucleic acid sequences. Together, the gRNA and the Cas9 induce double-stranded (DSB) DNA breaks at conserved sequences called proto-spacer adjacent motifs (PAM) (Jansen et al., 2002; Jinek et al., 2012). Each induced break activates the DNA repair machinery to resolve the DSB in one of two different manners: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is error prone and occurs with high efficiency, resulting in insertion or deletions (Indels) which can then disrupt or abolish the function of target genes. Conversely, HDR is error-free and uses an exogenous homology donor DNA sequence as the repair template. With HDR, desired sequences can be introduced into the DNA genome. It is precisely the ability of the CRISPR/Cas9
system to introduce changes in the DNA that makes this an invaluable tool in studying gene function as deletions in germ line cells generate inheritable transgenics. Above all, this method of gene silencing results in a true knock-out system that can be specific in nature. Despite the many advantages of CRISPR/Cas9, the approach is not without some issues. For instance, there is a potential for off-target DNA cleavage by mismatches of sgRNA with their target regions (Fu et al., 2013).

3.5 Summary

The development of transgenesis in mosquitoes has come a long way since the discovery of P elements in maize by Barbara McCintock. Since their use in drosophilid methods of germ-line transformation, transposons have advanced the field of insect transgenesis. These mobile DNA elements have in turn been adapted for transgene integration into the *Aedes* genome with the use of four transposons: *Hermes*, Mos1 (*mariner*), and *piggyBac*. While transposons appear to have large carrying capacities, site-specific recombinases are used as an alternative approach which permits transformations of much larger sequences and mitigates positional effect. An example of this approach is the use of the φC31 system in *Aedes aegypti*. Finally, the more recent rise of genome editing has allowed for targeted genetic integration by using DNA nucleases. Three examples of this editing approach are zinc-finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system.

These advances in mosquito transgenesis have developed a molecular genetics tool-box that includes transposon mediated gene transfer, site-specific
recombinases, and genome editing. In addition, bipartite systems such as the GeneSwitch system, which will be described in Chapter 6, are used in conjunction with these molecular approaches to perform elegant reverse genetic experiments that answer basic biology questions of mosquitoes.
Figure 13. Schematic of transgenesis development in *Aedes aegypti*. Class II transposable elements: *Hermes, Mos1 (mariner)*, and *piggyBac*. Site specific integrase system: serine integrase φC31 Custom-engineered nucleases: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat-associated endonucleases (CRISPR/Cas).
References


Chapter 4: 20-hydroxyecdysone stimulates juvenile biosynthesis by the \textit{corpora allata} of mosquito pupae

4.1 Abstract

Juvenile hormone III (JH) is synthesized by the \textit{corpora allata} (CA) and plays a key role in mosquito development and reproduction. JH titer decreases in the last instar larvae allowing pupation and metamorphosis to progress. As the anti-metamorphic role of JH comes to an end, the CA of the late pupa (or pharate adult) becomes again “competent” to synthesize JH, which plays an essential role orchestrating reproductive maturation. The molting hormone 20-hydroxyecdysone (20E) prepares the pupae for ecdysis, and would be an ideal candidate to direct a developmental program in the CA of the pharate adult mosquito. In the present study, we provide evidence that 20E acts as an age-linked hormonal signal, directing CA activation in the mosquito pupae. Stimulation of the inactive brain-\textit{corpora allata-corpora cardiaca} complex (Br-CA-CC) of the early pupa (24 hours before adult eclosion or -24 h) \textit{in vitro} with 20E resulted in a remarkable increase in JH biosynthesis, as well as increase in the activity of juvenile hormone acid methyltransferase (JHAMT). Addition of methyl farneosate but not farnesoic acid also stimulated JH synthesis by the Br-CA-CC of the -24 h pupae, proving that epoxidase activity is present, but JHAMT activity is missing. Separation of the \textit{corpora allata-corpora cardiaca} complex from the brain (denervation) in the -24 h pupae also activated JH synthesis. Our results suggest that an increase in 20E titer overrides an inhibitory effect of the brain on JH synthesis, phenocopying denervation. All together these findings provide
compelling evidence that 20E acts as a developmental signal increasing JHAMT activity that ensures proper reactivation of JH synthesis in the pharate adult mosquito.

4.2 Introduction

Periodic pulses of ecdysteroids (20E) and juvenile hormone (JH) orchestrate the timing of organism-wide developmental transitions in insects (Yamanaka et al., 2013; Riddiford, 2012). Juvenile hormone (JH) delays metamorphosis until larvae have attained an appropriate stage and size. At that point, a drop in JH secretion permits a metamorphic molt (Riddiford, 2012; Smykal et al., 2014). JHs are synthesized by the corpora allata (CA), a pair of endocrine glands with neural connections to the brain (Tobe and Stay, 1985). In Aedes aegypti mosquitoes the CA is inactive for most of the duration of the pupal stage (Nouzova et al., 2011; Rivera-Perez et al., 2014). As the anti-metamorphic role of JH comes to an end, the CA of the late pupa (or pharate adult) is reactivated and becomes “competent” to synthesize JH, which plays an essential role in coordinating reproductive maturation. (Klowden, 1997).

The activation of the CA after metamorphosis in mosquitoes is a fascinating and tightly regulated developmentally controlled process. The initiation of JH synthesis in pharate adult mosquito is partitioned into two temporally discrete steps: first a process of maturation during the last 6-8 hours of pupal stage prepares the CA gland to start synthesizing low amounts of JH (Rivera-Perez et al., 2014). At eclosion, a 2-fold increase in JH synthesis brings JH synthetic rates
to a value of 15-20 fmol/h, a threshold level required for the maximal expression of an early-posteclosion JH-dependent gene cluster (Zou et al., 2013; Riddiford, 2013). The two-step activation guarantees that a proper rise of JH synthesis concurs with adult eclosion. So we can define the activation of JH biosynthesis as a process “associated” to ecdysis, and its correct timing is critical. Factors involved in the initiation and scheduling of the ecdysis sequence, such as 20-hydroxyecdysone (20E) and ecdysis triggering hormone (ETH), are ideal to time CA activation with molt (Žitňan and Adams, 2012; Adams et al., 2013). We have previously defined the critical role of ETH acting as an allatotropic regulator of JH biosynthesis (Areiza et al., 2014).

Circulating levels of 20E in female mosquitoes increase in the last hours before adult eclosion (Supplemental Fig. 1), corresponding well with increases of synthesis and titer of JH (Margram et al., 2006; Rivera-Perez et al., 2014; Hernandez-Martinez et al., 2015); as well as increases of transcripts and activity of juvenile hormone acid methyl transferase (JHAMT), a key enzyme that transforms farnesoic acid (FA) into methyl farnesoate (MF) (Areiza et al., 2014). To further test the notion that 20E mediates the temporal control of CA maturation, we assayed its ability to trigger the premature activation of the CA of early pupae (24 h before adult eclosion or – 24 h). Indeed, in vitro stimulation with 20E of brain-corpora allata-corpora cardiaca complexes (Br-CA-CC) dissected from early pupae resulted in a significant increase of JH synthesis. We observed that CA from early pupae exhibited high levels of FA but little MF and undetectable levels of JH. Correspondingly, addition of FA failed to stimulate JH
synthesis by the Br-CA-CC complexes dissected from early pupae, while addition of MF resulted in a significant increase of JH synthesis. Consistent with this finding, stimulation with 20E resulted in an increase in the activity of JHAMT. We next assessed whether factors from the brain modulate JH synthesis in early pupae. Denervation resulted in a sharp increase in JH synthesis; suggesting that a factor from the brain inhibits JH synthesis in early pupa. All together these results support the conclusion that an increase in 20E titer in late pupae is sufficient to activate JH biosynthesis by overriding the effect of a brain inhibitory factor.

4.3 Materials and Methods
4.3.1 Insects

*A. aegypti* of the Rockefeller strain were reared at 28 °C and 80% humidity as previously described (Nouzova et al., 2011). Female pupae were collected at 30 min intervals as they molted from fourth instar larvae into the pupal stage. The duration of the pupal stage was determined to be 50 h (Areiza *et al.*, 2014).

4.3.2 Chemicals

20-hydroxyecdysone (20E) was provided by Sigma (St. Louis, MO). Stock of aqueous solutions of 20E were prepared at a concentration of 10⁻⁵ M and stored in aliquots at -80 °C. Farnesoic acid (FA) and methyl farnesoate (MF) were purchased from Echelon Biosciences (Salt Lake City, UT).

4.3.3 Dissections of *corpora allata* complexes
Female mosquito pupae were cold-anesthetized and corpora allata complexes were dissected in Aedes physiological saline (APS) (138 mM NaCl, 8.4 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 12 mM NaH₂PO₄, 12 mM Na₂HPO₄ and 42.5 mM sucrose) as previously described (Li et al., 2003a). Unless otherwise noted, preparations were of intact corpora allata-corpora cardiaca complexes connected to the brain and head capsule and are denoted as, Br-CA-CC complexes. “Denervated” CA-CC complexes are CA-CC separated from the brain (Nouzova et al., 2012).

4.3.4 Testing the effect of 20E on JH synthesis

Br-CA-CC complexes were dissected and incubated in the presence of different concentrations of 20E (10⁻⁵ to 10⁻⁷ M) at 32°C for 4 h in 150 µl of tissue culture media M-199 (Lavallette, NJ, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and 50 µM methionine. Controls were not treated with the peptide. Biosynthesized JH III was labelled with a fluorescent tag and analyzed by reverse phase high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) as previously described (Rivera-Perez et al., 2012).

4.3.4 Juvenile hormone acid methyltransferase activity assay

Five Br-CA-CC or CA-CC were dissected in APS and transferred to 100 µl of Tris-HCl buffer (50 mM, pH 7.4), sonicated in a water bath sonicator for 3 min, placed on ice for 1 min and centrifuged (13, 000 g, 10 min, 4 °C). Supernatants (crude extract) were collected. Juvenile hormone acid methyltransferase (JHAMF) activity in crude extracts was tested by adding farnesoic acid (FA) to a final concentration of 50 µM, and S-adenosyl methionine (SAM) to a final
concentration of 10 µM. Samples were incubated for 2 hours in a water bath at 37 ºC (Rivera-Perez et al., 2014). Reactions were stopped by adding 500 µl of hexane. Samples were vortexed for 1 min and centrifuged (13, 000 g, 10 min, 4 ºC). The organic phase was recovered and filtered into a new Eppendorf tube. Lastly, samples were dried with N₂ and stored at -20 ºC until analyzed. Conversion of FA into methyl farnesoate (MF) was determined by HPLC (Rivera-Perez et al., 2014).

4.3.5 Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software (San Diego, CA, USA). The results are expressed as means ± S.E.M. Significant differences (p< 0.05) were determined with a one tailed students t-test performed in a pair wise manner or by one-way ANOVA followed by Tukey’s test.

4.4 Results

4.4.1 20E stimulates JH synthesis by Br-CA-CC complexes dissected from early pupae

JH biosynthesis by Br-CA-CC complexes dissected from pupa -24 h before eclosion (early pupae) was significantly stimulated by in vitro treatment with 20E (Fig. 1). A series of 20E concentrations ranging from 10⁻⁵ to 10⁻⁷ M were tested. Only a concentration of 10⁻⁶ M prompted a significant increase of JH biosynthesis, while concentrations of 10⁻⁵ or 10⁻⁷ M were ineffective (Fig. 2).

To assess if the in vitro stimulatory effect of 20E was reversible, intact Br-CA-CC complexes were first incubated with or without 20E for a 2 h period. JH
synthesis was significantly stimulated by 20E (Fig. 3A). Afterwards, control and 20E-treated Br-CA-CC complexes were rinsed in fresh medium and incubated once more for 2 h in the presence or absence of 20E. While control glands that were never exposed to 20E remained inactive, those glands exposed to 20E during the first 2 h incubation period continued synthesizing high levels of JH independently of the presence or absence of 20E during the second incubation period (Fig. 3B).

4.4.2 20E stimulates JHAMT activity in the CA of early pupae

There are remarkable changes in the metabolic profiles of JH precursors and the activities of JH biosynthetic enzymes in the CA of early and late mosquito pupae (Rivera-Perez et al., 2014). We have detected high levels of most metabolites corresponding to the late step of JH synthesis in the CA of the early pupae, including farnesoic acid, but little methyl farnesoate and undetectable levels of JH (Fig. 4A). Correspondingly we have found significant activities of most of the enzymes of the JH-branch, including farnesal dehydrogenase (FALDH) that converts FA into MF, but not detectable activity of JHAMT (Fig. 4B).

To assess the effect of addition of exogenous precursors on JH synthesis, Br-CA-CC were dissected from early pupae (-24 h) or late pupae (-6 h) and incubated in vitro in the presence of farnesoic acid (FA) and methyl farnesoate (MF). The addition of these two late JH precursors to the incubation media had different stimulatory effects on the synthesis of JH by the Br-CA-CC complexes dissected from early or late pupae. While MF efficiently increased JH
synthesis when added to both glands, FA only stimulated JH synthesis when added to the Br-CA-CC complexes dissected from late pupae (Fig 5).

The results of these experiments imply that the CA from early pupa (-24 h) lacks JHAMT activity and is unable to convert FA into MF but presents epoxidase activity and efficiently metabolizes MF into JH. To test this hypothesis Br-CA-CC were dissected from early pupae (-24 h) and incubated in vitro for 4 h in the presence of 20E. Ecdysteroid-treatment resulted in a significant increase in JHAMT activity (Fig. 6).

4.4.3 Denervation activates JH synthesis in early pupa

To determine whether factors from the brain modulate JH synthesis in early pupae (-24 h), we measured in vitro JH biosynthesis by denervated glands (CA-CC). Denervation resulted in a sharp increase in JH synthesis (Fig. 7); suggesting that a factor from the brain inhibits JH synthesis in early pupa.

4.5 Discussion

The biosynthesis of JH-III in A. aegypti involves 13 sequential enzymatic steps. The early steps follow the mevalonate pathway up to the formation of farnesyl diphosphate (FPP); in the late steps, FPP is transformed sequentially to farnesol, farnesal, FA, MF and ultimately JH III (Rivera-Perez et al., 2014). JH biosynthesis is controlled by the rate of flux of isoprenoids in the pathway, which is the outcome of a complex interplay of changes in transcripts, enzyme activities and metabolites (Nouzova et al., 2011; Rivera-Perez et al., 2014). Molecules responsible for the activation, modulation and suppression of JH synthesis in female mosquitoes include ETH (Areiza et al., 2014), allatostatin-C (AST-C)
(Nouzova et al., 2015), insulin (Perez-Hedo et al., 2012, 2013) and allatotropin (AT) (Li et al., 2003b). The activities of these factors are linked to developmental and nutritional signals (Noriega 2014). JH is therefore an important part of a transduction mechanism that connects developmental changes and nutritional status with the activation of specific physiological events during reproduction (Noriega, 2004; Clifton and Noriega, 2011, 2012).

We have earlier reported that brain-corpora allata-corpora cardiaca complexes (Br-CA-CC) dissected from mosquito pupae up to 10 h prior to adult eclosion do not synthesize detectable amounts of JH, and are insensitive to ETH stimulation (Areiza et al., 2014). On the contrary, addition of ETH to Br-CA-CC complexes dissected from late pupa ranging from 8 to 2 h before eclosion show significantly higher levels of JH biosynthesis and JHAMT activity (Areiza et al., 2014).

Our studies are unraveling a scheme of interconnected molecular events explaining how 20E and ETH instruct the timing of CA maturation and activation in late pupae of mosquitoes. Pharate adult mosquitoes exhibit marked temporal changes in JH synthesis and JHAMT activity, which match well with 20E dynamics. The genes encoding ETH and ETH receptors (ETHR) are also under tight regulation by 20E (Žitňan and Adams, 2012). That suggested that an increase in 20E titer might provide a timing cue to turn on the activating program in CA in the late pupae. We tested this hypothesis in a series of experiments. Indeed, 20E produced precocious increases in JH biosynthesis. Therefore, manipulating 20E titer was sufficient to trigger CA maturation.
Remarkably, the stimulatory effect of 20E occurred in a narrow concentration range that might mimic the 20E rise in the late pupae; this surge of steroids regulates ETH receptor expression in the mosquito CA providing the gland with the competence to respond to ETH (Areiza et al., 2014), as well as increases ETH levels in Inka cells (Dai and Adams, 2009; Areiza et al., 2014). On the other hand, declining steroid levels are required for secretory competence of Inka cells (Kingan et al., 1997; Kingan and Adams, 2000). Small concentration windows of rising and declining 20E levels provide a molecular framework to explain how systemic hormonal control coordinates tissue specific programs of differentiation with developmental timing.

In contrast to a JH modulator such as AST-C that exerts a strong, rapid and reversible *in vitro* inhibition of JH synthesis that can be overridden by removing the peptide (Nouzova et al., 2015), the *in vitro* stimulatory effect of 20E was irreversible and could not be overturned by washing away the hormone. Nutritional modulators such as AST-C and insulin control the availability of precursors, such as cytoplasmic acetyl-CoA that sustains JH synthesis in the CA of mosquitoes, without affecting the synthetic potential of the CA (Nouzova et al., 2015). On the contrary, developmental regulators such as ETH and 20E, tend to modulate the activity of key enzymes like JHAMT (Areiza et al., 2014). Enzyme levels need to surpass a minimum threshold to achieve a net flux of precursors through the JH biosynthetic pathway; an increase in JHAMT enzymatic activity is critical for the increases in JH biosynthesis in pharate adult and newly eclosed females (Rivera-Perez et al., 2014; Areiza et al., 2014). *In vitro* incubation of Br-
CA-CC with 20E was sufficient to induce a remarkable increase in JHAMT activity. The increase of 20E titer in late pupae might act as a checkpoint that commits the CA irreversibly. There are many examples of physiological processes irreversibly initiated by 20E (Stieper et al., 2008).

We have previously described stimulatory and inhibitory effects of brain factors in mosquitoes. Denervation prevents a 10-fold activation of JH synthesis that occurs 12 h after adult eclosion (Hernandez-Martinez et al., 2007; Rivera-Perez et al., 2014). In contrast, denervation causes a significant increase in JH synthesis in sugar-fed and blood-fed females (Li et al., 2004). Distinct neural modulators likely mediated these effects. For example, AST-C and AT are present in the brain of A. aegypti (Hernandez-Martinez et al., 2005). They both modulate JH synthesis in vitro (Li et al., 2004; Li et al., 2006) and their receptors are expressed in the CA (Mayoral et al., 2010; Nouzova et al., 2012).

Studies on Manduca sexta larvae also suggested that 20E controls JH synthesis acting indirectly via the Br-CC. Dose-response analyses revealed that 20E had a stimulatory effect on JH synthesis by the CA, but only when the glands were complexed with the brain-corpora cardiaca (Whisenton et al., 1985; LaVern et al., 1987). We observed that separation of the CA from the brain (denervation) resulted in a remarkable activation of JH synthesis in early pupae; suggesting that the activity of the CA is in fact actively repressed by the brain. The fact that denervation phenocopies the result of 20E stimulation prevented us to test a direct effect of the hormone on CA-CC preparations.
Ecdysteroid signaling in insects is transduced by a hetero-dimer of ecdysteroid (EcR) and ultraspireacle (USP) nuclear receptors (Hill et al., 2013), and both receptors are expressed in the CA of the pupae (Supplemental Fig. 2). Although our results argue for a brain-dependent effect of 20E on JH synthesis, with the steroid overriding the allatostatic role of the brain; we cannot rule out a direct effect of 20E on the CA. In fact, 20E has been already proposed as an important regulator of the transcription of JH biosynthetic enzymes in Bombyx mori (Gu and Chow, 1996; Kaneko et al., 2011).

4.6 Conclusions

Taking advantage of the recent development of a HPLC-FD protocol that allows the measurement of JH and its precursors, as well as the enzymatic activities of the biosynthetic enzymes, we set out to test hypothesis that 20E plays a key role on the reactivation of the CA in the late pupae of mosquitoes. Our in vitro approaches combined with the analyses of precursors and enzymes in the isolated CA provided evidence that:

- The CA of early pupae has a sizeable amount of FA, but it is deficient in JHAMT activity and MF.
- 20E stimulates JHAMT activity that catalyzes the conversion of FA into MF.
- The stimulatory effect of 20E is irreversible and occurred only in a narrow concentration range,
- 20E acts as a “derepressor”, overriding the action of an inhibitory brain factor.
Before the rise of 20E titers in late pupa, the CA activation program has been progressively assembled, but it is kept on hold through expression of a brain repressor. The increase of the 20E titer provides temporal cues for the execution of a CA maturation program.

4.7 Acknowledgments
This work was supported by NIH Grant No AI 45545 to F.G.N.

4.8 Author contributions
MA, MN, CRP and FGN developed the concepts and approaches. MA, MN and CRP performed experiments. MA, MN, CRP and FGN did data analysis. MA, MN, CRP and FGN prepared and edited the manuscript prior to submission.
Figure 14 JH Effect of 20E on JH synthesis by Br-CA-CC complexes from “early pupae”.

JH biosynthesis by Br-CA-CC complexes incubated by 4 h without (control) and with (+20E) addition of 20E (10⁻⁶ M) was assayed *in vitro* using the HPLC-FD detection method. Each data point represents the mean ± S.E.M. of at least 25 independent determinations of groups of 4 Br-CA-CC complexes dissected from -24 h pupae. Asterisks denote significant differences (unpaired t-test; *** P ≤ 0.001).
Figure 15 Dose-response effect of 20E.

The effect on JH synthesis of 20E concentrations ranging from 10^{-5} to 10^{-7} M were tested on BR-CA-CC complexes dissected from -24 h pupa.
**Figure 16** Br-CA-CC were incubated for two consecutive 2 h periods. A) During the first 2 h, Br-CA-CC complexes were incubated either in the presence or absence of 20E ($10^{-6}$ M). After 2 h, glands were rinsed in fresh culture medium. B) During the second 2 h period, control and previously treated glands were incubated in fresh culture medium with and without 20E. Each data point represents the mean ± S.E.M. of three independent determinations of three Br-CA-CC complexes. Asterisks denote significant differences (unpaired t-test; *** P ≤ 0.001). Different letters above the columns indicate significant differences among treatments (ANOVA P<0.05, with Tukey’s test for multiple comparison).
Figure 17. Metabolite pool sizes and enzymatic activities in CA of the early pupae. A) Relative levels of endogenous concentrations of farnesoic acid (FA) and methyl farnesoate (MF), and juvenile hormone biosynthesis rates in CA of early (-24 h) and late (-6 h) pupae. B) Farnesal dehydrogenase (FALDH) and juvenile hormone acid methyl transferase (JHAMT) enzymatic activities in CA extracts of early (-24 h) and late (-6 h) pupae. Bars represent the means ± SEM of three independent replicates of three groups of 3 CA.
Figure 18 Effect of stimulation with precursors on JH biosynthesis

The effect of addition of precursors on JH biosynthesis was evaluated on Br-CA-CC complexes incubated in culture medium M-199 alone (control) or with the addition of 200 μM of FA (farnesoic acid) and MF (methyl farnesoate). A) Br-Ca-CC dissected from early pupae (-24 h). B) Br-Ca-CC dissected from late pupae (-6 h). Each data point represents the means ± S.E.M. of 2-4 independent biological replicates of three Br-CA-CC complexes. (ANOVA P<0.05, with Tukey’s test for multiple comparison).
Figure 19 Effect of 20E on JHAMT enzymatic activity.

Br-CA-CC complexes were incubated by 4 h without (control) and with (+20E) addition of 20E (10-6 M). Consequently CA-CC extracts were prepared and JHAMT activity was measured. Each data point represents the mean ± S.E.M. of three independent determinations of five CA complex. Asterisk denotes significant difference (unpaired t-test; *** P ≤ 0.001).
Figure 20 Effect of denervation on JH synthesis.

Br-Ca-CC and CA-CC complexes were dissected from -24 h pupae and incubated in vitro for 4 h. JH biosynthesis was assayed using the HPLC-FD detection method. Each data point represents the mean ± S.E.M. of 3 independent determinations of 4 complexes. Asterisk denotes significant difference (unpaired t-test; *** P ≤ 0.001).
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Chapter 5: 20-Hydroxyecdysone acts as an allatotropic factor for the reactivation of the CA post blood feeding

5.1 Abstract

In *A. aegypti*, the *corpora allata* (CA) alternates between distinct physiological states which are linked to reproductive physiology. Like JH, the ecdysteroid 20-hydroxyecdysone (20E) is a key hormonal regulator in both development and ovarian maturation and has been proposed as an allatoregulator in other insects. Previously, we have shown that 20E is an allatoregulatory factor in the pharate adult (pupae) that directs the activation of the CA in synchrony with adult ecdysis. In the present study, we demonstrate that 20E plays a role in the reactivation of the CA in the blood-fed female. Our findings show that *in vitro* stimulation with 20E at 24h post blood feeding reactivates the CA. Additionally, the application of 20E results in an increase of farnesal dehydrogenase (FALDH) activity. Consistent with this finding, addition of farnesoic acid (FA) stimulates JH synthesis in blood fed females underscoring the effect of 20E on FALDH activity. Separation of the brain from the retrocerebral complex (CA-CC) in the 24h blood fed female, also increases JH synthesis, suggesting a factor from the brain represses CA activity. Our results suggest that an increase 20E overrides an inhibitory effect of the brain on JH synthesis, phenocopying decapitation. Silencing of EcRs by RNA interference (RNAi) targeting both isoforms of the receptor for 20E did not further elucidate the role of 20E on JH synthesis. Taken together, the data suggest a continuing
allatotropic role of 20E in the CA of the blood fed female which mediates the reactivation of the CA in preparation for the second gonadotrophic cycle.

5.2 Introduction

In the adult female mosquito, regulation of JH biosynthesis is especially nuanced during the vitellogenic cycle. In particular, the transduction of nutritional signals determines the progression of oogenesis. The CA-CC complex of A. aegypti expresses receptors for various regulatory molecules including, allatotropins (AT), allatostatins (AS), insulin, ecdysis triggering hormone (ETH), and 20E (Mayoral et al., 2010; Nouzova et al., 2012; Perez-Hedo et al, 2012; Areiza et al., 2014, 2015).

Previous investigations have shown that 20E regulates JH synthesis in the larva of M. sexta and B. mori (Whisenton et al., 1985; 1987; Kaneko et al., 2011). In mosquitoes, our work has shown that ETH and 20E are repurposed to other functions, namely as allatoregulatory factors in the pupal stage of mosquitoes (Areiza et al, 2014; 2015). In D. melanogaster adults, a recent study has revealed cross-talk between 20E, ETH and JH that ensures reproductive success in adults (Meiselman et al., 2017). Central to the elegant work of Meiselman and colleagues is the notion that gonadotropin hormones like JH and 20E are usually studied in isolation. Rather, these hormones should be viewed within an endocrine context, particularly considering that JH and 20E work in conjunction to orchestrate progression of oogenesis in mosquitoes. The gonotrophic cycle is directed by JH and 20E through a biphasic cyclic fluctuation of their levels that is
mediated by nutritional status (Briegel, 1990; Noriega, 2004). JH controls the first two periods of the vitellogenic cycle, previtellogenesis and ovarian resting stage. While 20E mediates vitellogenesis and subsequent completion of egg development. After a blood meal, JH levels drop and 20E levels rise allowing for the female mosquito to enter the synthetic phase of the vitellogenic cycle. The action of 20E in vitellogenesis is mediated by the transduction of nutritional signals that include, acquired amino acids from the blood meal and insulin (Roy et al., 2016). During vitellogenesis, 20E matures the developing oocyte but also stimulates the separation of the next secondary follicle. The levels of 20E begin to drop and reactivation of the CA activity begins. This rise of biosynthetic activity of the gland is essential to ensure the transition to another gonadotrophic cycle.

Here we show that *in vitro* stimulation with 20E at 24h post blood feeding, reactivates the CA at a time when the CA is in a suppressed state. Moreover, the application of 20E increases the enzymatic activity of FALDH, an enzyme that is inactive in blood fed females. Moreover, addition of FA, a precursor of JH biosynthesis, to BR-CA-CC complexes of 24h PBM stimulates JH synthesis. The stimulatory effect of FA further supports the importance of the enzymatic activity of FALDH in the reactivation of the post blood-fed female. In insects, the allatoregulatory role of the brain is widely accepted. Indeed, previous studies that have looked at the role of 20E on JH synthesis have found that the brain is inhibitory to biosynthesis (Whisenton *et al.*, 1985, 1987; Li *et al.*, 2004; Kaneko *et al.*, 2011). To discern if the brain plays an allatoregulatory role in the blood fed female, we performed separations of the brain from the retrocerebral complex.
We found that denervation also increases JH synthesis, suggesting a factor from the brain represses CA activity. Our results suggest that an increase 20E overrides a brain inhibition on the CA, phenocopying decapitation. Silencing of EcRs by RNA interference (RNAi) targeting both isoforms of the receptor for 20E did not further elucidate the role of 20E on JH synthesis. Taken together, the data suggest a continuing allatotrophic role of 20E in the CA of the blood fed female which mediates the reactivation of the CA in preparation for the second gonadotrophic cycle.

5.3 Materials and Methods

5.3.1 Insects

*Aedes aegypti* of the Rockefeller strain were reared at 28 °C and 80% humidity as previously described (Nouzova *et al*., 2011). Adult mosquitoes were offered a cotton pad soaked in 3% sucrose solution. Four-day old female mosquitoes were blood fed by providing pig blood equilibrated to 37°C with ATP added to a final concentration of 1mM as previously described (Nouzova *et al*., 2011).

5.3.2 Chemicals

The ecdysteroid, 20-hydroxyecdysone (20E) was provided by Sigma (St. Louis, MO). Stock of aqueous solutions of 20E were prepared at a concentration of 10-4 M and stored in aliquots at -20 °C. Farnesal (FAL) and Farnesoic acid (FA) were purchased from Echelon Biosciences (Salt Lake City, UT). FA and FAL were dissolved in MeOH in 10ug/ul to make a stock. Stockls were diluted in
medium M-199 to the desired concentration, (MetOH was evaporated and the FA or FAL were re-dissolved in medium M-199, sonicated for 3 min).

5.3.3 Dissections of corpora allata complexes

Female mosquitoes were cold-anesthetized and corpora allata complexes were dissected in Aedes physiological saline (APS) (138 mM NaCl, 8.4 mM KCl, 4 mM CaCl$_2$, 2 mM MgCl$_2$, 12 mM NaH$_2$PO$_4$, 12 mM Na$_2$HPO$_4$ and 42.5 mM sucrose) as previously described (Li et al., 2003a). The preparations consisted of intact corpora allata-corpora cardiaca complexes connected to the brain and head capsule and are denoted as Br-CA-CC complexes.

5.3.4 Testing the effect of 20E on JH synthesis

To examine the effect of 20E on JH biosynthesis, Br-CA-CC complexes were dissected and incubated in the presence of 20E at a concentration of $10^{-6}$ M at 32°C for 4 h in 150 µl of tissue culture media M-199 (Lavallette, NJ, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and 50 µM methionine. Controls were not treated with the peptide. Biosynthesized JH III was labelled with a fluorescent tag and analyzed by reverse phase high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) as previously described (Rivera-Perez et al., 2012).

5.3.5 Farnesal dehydrogenase activity assay

Glands were dissected in APS and transferred to 100 µl of Tris-HCl buffer (50 mM, pH 7.4), sonicated in a water bath sonicator for 3 min, placed on ice for 1 min and centrifuged (13,000 g, 10 min, 4 °C). Supernatants (crude extract) were collected. Farnesal dehydrogenase (FALDH) activity in crude extracts was
tested by adding farnesal (FAL) to a final concentration of 50 µM, and S-adenosyl methionine (SAM) to a final concentration of 10 µM. Samples were incubated for 2 hours in a water bath at 37 °C (Rivera-Perez et al., 2013). Reactions were stopped by adding 500 µl of hexane. Samples were vortexed for 1 min and centrifuged (13, 000 g, 10 min, 4 °C). The organic phase was recovered and filtered into a new Eppendorf tube. Conversion of FAL into farnesoic acid (FA) was determined by HPLC (Rivera-Perez et al., 2014).

5.3.5 RNAi knock-down experiment

Double-stranded RNA (dsRNA) was designed against a common region of both EcR transcript variants (EcrA and EcrB). As a control, dsRNA was synthesized encoding the Yellow Fluorescent Protein (YFP). For the generation of EcR and YFP dsRNAs, target sequences were amplified by PCR using the primers included in Supplemental Table 1 (Figure 6). dsRNAs were produced by means of in vitro transcription with the T7 RNA polymerase using the MEGAscript T7 kit (Ambion, Austin, TX, USA) as previously described (Pérez-Hedo et al., 2013). Newly eclosed female mosquitoes were cold-anesthetized and RNAi mediated knock-down of EcR receptors was accomplished by intrathoracic injection of 1.8 µg of EcRi or YFPi dsRNA using a Drummond Nanoject II microinjector and a micromanipulator. After injections mosquitoes were maintained in the insectary under normal rearing conditions and fed 3% sucrose solution for 2 days. On the third day after injections, they were mated and blood fed. The effect of dsRNA was evaluated 24h after the blood-meal.
5.3.6 Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software (San Diego, CA, USA). The results are expressed as means ± S.E.M. Significant differences (p< 0.05) were determined with a one tailed students t-test performed in a pair wise manner or by one-way ANOVA followed by Tukey’s test.

5.4 Results

5.4.1 20E stimulates JH synthesis by Br-CA-CC complexes in the blood fed female

Juvenile hormone biosynthesis by Br-CA-CC complexes dissected from 24h blood fed females was significantly stimulated by *in vitro* treatment with 20E, at a physiological concentration of 10^{-6} M. To assess the stimulatory effect of 20E, intact Br-CA-CC complexes were incubated *in vitro* with or without 20E for a 2 h period. JH synthesis was significantly stimulated by 20E (Figure 21). Afterwards, control and 20E-treated Br-CA-CC complexes were rinsed in fresh medium and incubated once more for 2 h in the presence or absence of 20E.

5.4.2 20E stimulates FALDH activity in the CA of the blood fed female

To evaluate the effect of 20E on the enzymatic activity of FALDH, Br-CA-CC were dissected from blood fed females (24h PBM) and incubated *in vitro* for 4 h in the presence of 20E. Ecdysteroid-treatment resulted in a significant increase in FALDH activity (Figure 22). To assess the effect of addition of exogenous precursors on JH synthesis, Br-CA-CC were dissected females 24h PBM and incubated *in vitro* in the presence of FA. The addition of FA to the incubation media had a stimulatory effect on the premature synthesis of JH by the Br-CA-
CC complexes dissected (Figure 23). The results of these experiments imply that the CA from a blood fed female lacks FALDH activity and thus is unable to convert FAL into FA. However, the CA does present JHAMT activity, and epoxidase (EPOX) which is capable of catalyzing the FA and MF leading to JH synthesis.

5.4.3 Separation of the brain from the retrocerebral complex increases JH synthesis

To determine whether factors from the brain modulate JH synthesis at 24h PBM, we measured in vitro JH biosynthesis by denervated glands (CA-CC). Denervation resulted in a sharp increase in JH synthesis (Figure 24); suggesting that a factor from the brain inhibits JH synthesis.

5.4.4 RNAi-mediated depletion of EcR receptor mRNA in blood fed adult does not result in a reduction of JH synthesis by BR-CA-CC

Newly eclosed female mosquitoes were injected with either a control dsRNA for YFP or dsRNA targeting the two isoforms of EcR receptors (EcR-A and EcR-B). Three days after injections, they were mated and blood fed. The effect of dsRNA was evaluated 24h after the blood-meal. To verify RNAi-mediated in vivo depletion of the ecdysone receptors, abdomens from blood fed females (24 h PBM) were used to assess transcript levels. While EcR transcript levels were significantly decreased (69%) when compared to YFP controls (Figure 25). However, there were no statistically significant differences in JH synthesis between YFP controls and RNAi silenced females.
5.5 Discussion

Previously we have shown that in the pharate pupae, 20E acts as a developmental signal that ensures the activation of the CA in the last hours of the pupal stage (Areiza et al., 2015). Activation of the pharate CA results in the synthesis of low basal levels of JH in the first hours after emergence; JH levels continue to steadily rise to a peak at 12 h (River-Perez, et al., 2014). In the newly eclosed female, JH is necessary to complete the previtellogenic preparatory period of the primary follicles as well as in mediating adaptation for blood feeding physiology (Shapiro and Hagedorn, 1982; Noriega et al., 1997). Remarkably, the primary follicles remain in a state of “dynamic” arrest until the ingestion of a blood meal, after which 20E mediates vitellogenesis (Hagedorn, 1974; Klowden 1997).

As an anautogenous mosquito, A. aegypti requires a blood-meal to complete vitellogenesis. JH and 20E regulate the progression of the gonotrophic cycle through a biphasic cyclic fluctuation of their levels that is mediated by nutritional status (Briegel, 1990; Noriega, 2004). For instance, blood feeding stimulates a rise in the circulating titers of 20E (Hagedorn et al., 1975). Conversely, the ingestion of a blood meal results in suppression of the CA during the first 24 hours that is critical for the completion of vitellogenic development of the primary follicles. However, 72 h after a blood meal, the CA transitions from physiological state of “suppressed” JH biosynthesis to one of “reactivation. This transition in CA activity is necessary for successful ovarian development and the initiation of the second gonotrophic cycle. Consequently, CA activity must be tightly regulated to ensure proper progression of the gonotrophic cycle.
Pulses in ecdysteroid titers are critical in ensuring proper developmental timing of behavior and physiological changes in insects (Ashburner et al., 1974; Riddiford, 1993; Cherbas and Cherbas, 1996; Riddiford and Cherbas, 2000). Moreover, previous studies on Drosophila and A. aegypti have shown that the nature of the 20E pulses, whether they are high or low, elicit different responses in tissues adding to the versatility of action of 20E (Thummel and Karim, 1992; O’Connor et al., 2014). In the female mosquitoes, pulses of 20E are nutritionally modulated and are perfectly poised to serve as a signal denoting the end of vitellogenesis and the reactivation of the CA in preparation for the second gonadotropin cycle.

After a blood meal, JH titers begin to rapidly decrease, reaching their lowest levels (0.6 fmol/female) at 24h post blood meal (Hernandez-Martinez et al., 2015). Additionally, analysis of enzymatic activities shows that at this time, the biosynthetic activity of farnesal dehydrogenase (FALDH) is not detectable, while juvenile hormone acid methyltransferase (JHAMT) activity is significant (Rivera-Perez et al., 2014). To test the notion that 20E mediates the activation of the suppressed gland in blood fed females, we assayed its ability to trigger the premature activation of the CA at 24h PBM. Our findings show that in vitro stimulation with 20E at 24h post blood feeding prematurely reactivates the gland suggesting an allotropic role for the ecdysteroid. Consistent with these findings, stimulation of 20E results in an increase of FALDH activity. At 24h PBM there are changes in the metabolite pool sizes of JH branch that reflect decreased enzymatic activity (Hernandez-Martinez et al., 2015). For example, the CA of
blood fed females exhibit high levels of FAL yet produce little FA. Accordingly, we found that addition of FA stimulates JH synthesis in blood fed females. To try to further our understanding of the stimulatory effect of 20E in the CA we performed RNAi dependent knockdown of EcRs targeting both isoforms of the receptor. Silencing of EcRs transcripts was verified using real time q-PCR and found to be 69%. In spite of the efficiency in silencing, it did not correlate with a reduction in JH synthesis. This is not entirely surprising due to the systemic nature of silencing; tissues used for q-PCR were abdomens and it is hard to discern if knockdown of the receptors in the CA was sufficient. In *A. aegypti*, EcR receptors are expressed differentially in various tissues such as the CA, midgut, salivary glands, ovary, and fat body (Cho et al., 1995; Wang et al., 2002, 2006; Parthasarathy and Palli, 2007). Moreover, the action of 20E via its receptors mediates several biological processes including, apoptotic responses, morphogenetic, physiological, behavioral and reproductive. Making it is difficult to assess how systemic receptor silencing may be reflected on JH synthesis by the CA.

There is a possibility that factors from the brain could modulate JH synthesis. We assessed this by denervating or separating the brain from the CA-CC and evaluating the effect on JH levels. We found that decapitation of 24h blood-fed females, results in premature reactivation of JH synthesis implying that 20E is mediating the suppression of brain factors on JH synthesis after blood-feeding. Taken together, the findings in this study show that in the blood fed
female 20E acts as a stimulatory effector of JH synthesis which mediates the reactivation of the CA in preparation for the second gonadotrophic cycle.

5.6 Conclusions

Our in vitro approaches combined with data of previous analyses of precursors and enzymatic activities in the CA before and after blood feeding provide evidence that at 24h post blood feeding, the CA has a sizeable amount of FAL, but it is deficient in FALDH. The inactivity of FALDH proves to be important as it influences the flux of precursors through the JH pathway and is reflected in the suppressed state of the CA in blood fed females. Our in vitro approach at 24h post blood feeding found that 20E treatments were able to initiate JH biosynthesis in the inactive CA prematurely. Moreover, addition of FA to 24h blood fed CA complexes in vitro increases JH synthesis. In this study, 20E stimulates FALDH activity that catalyzes the conversion of FAL into FA.

Surgical separation of the brain from the CA-CC complexes results in premature synthesis of JH suggesting an inhibitory role of the brain in CA biosynthesis. However in vitro treatments with 20E appears to override the possible brain mediated inhibition. RNAi targeting of 20E receptors (EcR) did not decrease JH synthesis; an in vivo approach would better discern the role of 20E in the reactivation of the CA post blood feeding.
Figure 14. Effect of 20E on JH synthesis by Br-CA-CC complexes from blood fed females. JH biosynthesis by Br-CA-CC complexes incubated for 2 h without (control) and with (+20E) addition of 20E (10−6 M) was assayed in vitro using the HPLC-FD detection method. Each data point represents the mean ± S.E.M. of 25 independent determinations of groups of 4 Br-CA-CC complexes dissected from 24h blood fed females. Asterisks denote significant differences (unpaired t-test; *** P ≤ 0.001 ).
Figure 15. Effect of 20E on FALDH enzymatic activity. Br-CA-CC complexes were incubated for 2 h without (Control) and with (+20E) addition of 20E (10^{-6} M). Consequently CA-CC extracts were prepared and FALDH activity was measured. Each data point represents the mean ± S.E.M. of three independent determinations of five CA complexes. Asterisk denotes significant difference (unpaired t-test; * P ≤ 0.0161).
Figure 16. Effect of stimulation with precursors on JH biosynthesis. The effect of addition of precursors on JH biosynthesis was evaluated on Br-CA-CC complexes incubated in culture medium M-199 alone (Control), 20E, or with the addition of 200 μM of FA. Each data point represents the means ± S.E.M of 2 independent biological replicates of three Br-Ca-CC complexes. (ANOVA P<0.05, with Tukey’s test for multiple comparison.)
Figure 17 Effect of denervation on JH synthesis. Br-CA-CC and CA-CC complexes were dissected from blood fed females (24h PBM) and incubated in vitro for 2 h. JH biosynthesis was assayed using the HPLC-FD detection method. Each data point represents the means ± S.E.M. of two independent determinations of 4 complexes. Asterisk denotes significant
Figure 18. Effect of EcR RNAi on 24h blood fed females. EcRs RNA levels were evaluated in abdomens. Newly eclosed female mosquitoes were injected with either a control dsRNA for YFP or dsRNA targeting the two isoforms of EcR receptors (EcR-A and EcR-B). Three days after injections, they were mated and blood fed. Each data point represents the means ± S.E.M. of 6 biological replicates. Asterisks denote significant difference (unpaired t-test, ** P≤0.0051).
**Figure 19.** Supplemental table of primers used to generate dsRNA templates for use in RNAi mediated silencing of EcR receptors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Template</th>
<th>Primer forward (5' → 3')</th>
<th>Primer reverse (5' → 3')</th>
<th>Note</th>
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<td>GGTACCAGGGACGGGTACGAG</td>
<td>GCGAACTCCAGATTAGTG</td>
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</tr>
<tr>
<td>EcR</td>
<td>A. aegypti cDNA</td>
<td>taatacagctcactatagggGTACCAGGA</td>
<td>taatacagctcactatagggGC GAACCTCCAGATTAGTG</td>
<td>Addition of T7 promoter</td>
</tr>
<tr>
<td>YFP</td>
<td>pEYFP-N1 Clontech</td>
<td>AACCCGATCGAGCTGA</td>
<td>TCAGGGCGGACTGGTA</td>
<td>PCR of target sequences</td>
</tr>
<tr>
<td>YFP</td>
<td>pEYFP-N1 Clontech</td>
<td>TAAATAGACTCAGACTATAGGGAAACGCA</td>
<td>TAATACGACTCAGACTATAGG GTCAGGGCGGACTGGTA</td>
<td>Addition of T7 promoter</td>
</tr>
</tbody>
</table>
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Chapter 6: Conclusions and Future Directions

6.1 Conclusions

*Aedes aegypti* mosquitoes has evolved in proximity to humans; they are highly anthropophilic in their feeding preferences (Powell *et al.*, 2013; Brown *et al.* 2014, McBride *et al.*, 2014). Their commensal habits make them especially suited to be vectors of arboviral diseases that afflict humans. *Aedes aegypti* mosquitoes are the principal vectors of four important diseases, yellow-fever, dengue, Zika, and Chikungunya. These viral diseases are major burden to public health worldwide and are responsible for considerable loss of productivity and human life. Currently a commercially available vaccine exists only for yellow fever. And therapeutics are not readily available to treat patients. In the absence of vaccines and effective treatments for these infections, vector control strategies offer the best prevention for outbreaks. The geographical expansion of vector range due to urbanization, minimization of eradication programs, and climatic changes increase the susceptibility of immunologically naïve populations encountering emerging tropical diseases (Jansen and Beebe, 2010). Accordingly, expanding our knowledge on basic vector biology is critical in developing novel and effective targeted vector control strategies. The regulation of biosynthesis of hormonal factors that mediate development and reproduction like juvenile hormone are attractive targets of research. The primary focus of this work was to elucidate how 20E might act as a regulatory effector of JH biosynthesis. The aim of this dissertation was to elucidate the role of 20E in
mediating the physiological transitions of CA activity in the pupa and the adult female. The specific aims were (1) to investigate the role in the activation of the late pupal CA and to (2) evaluate if 20E plays a role in the reactivation of JH synthesis in blood-fed females. Chapters four and five respectively address these aims.

In the last hours of the pupal stage, female *A. aegypti* mosquitoes have a small rise in circulating 20E levels that provide temporal cues for the execution of ecdysis that parallel an increase of synthesis and titer of JH (Hernandez-Martinez *et al.*, 2015; Margam *et al.*, 2006). Additionally, there is an increase in both transcription and enzymatic activity of juvenile hormone acid methyl transferase (JHAMT), a key enzyme that transforms farnesoic acid (FA) into methyl farnesoate (MF) (Rivera-Perez *et al.*, 2014). Previously, we have shown that factors involved in coordinating the ecdysis sequence, such as ecdysis triggering hormone (ETH), are ideal to time CA activation with the adult molt (Areiza *et al.*, 2014). Transcriptional activation of ETH and its cognate receptors are under regulation by 20E (Zitnan and Adams, 2012), suggesting a role of 20E in the maturation of the pharate adult (late pupa) CA. In chapter four, we demonstrate that 20E acts as a developmental hormonal signal that directs CA activation at the end of the pupal stage (Areiza *et al.*, 2015). We evaluated *in vitro* the role of 20E on JH synthesis by the mosquito CA of early pupae (24 h before adult eclosion or −24 h). Stimulations with 20E of intact retrocerebral complexes (Br-CA-CC), result in premature JH synthesis. It is worth mentioning that in the early pupae, the CA is inactive, transcript levels for most JH biosynthetic enzymes are
low, and there is substantial amount of farnesoic acid (FA), the penultimate precursor in the JH biosynthetic pathway. We assessed the effect of addition of exogenous precursors on JH synthesis and found that addition of FA did not stimulate JH synthesis in the early pupae; while addition of MF did increase JH synthesis. These findings are consistent with the observed increase in JHAMT activity in 20E treated CA. Lastly, we assessed the effect of JH synthesis when the brain was separated from the CA. Separation of the brain resulted in a sharp increase of JH synthesis in the early pupa. Taken together, these results suggest that the increase of 20E seen in the late pupae activates JH synthesis by inhibiting a neural repressor of JH synthesis. Moreover, the in vitro stimulatory effect of 20E was irreversible, supporting its role as developmental signal for the execution of a CA maturation program.

For anautogenous mosquitoes like A. aegypti, a blood-meal is required to complete oogenesis and results in suppression of CA activity. For successful ovarian development, the CA transitions from physiological state of "suppressed" JH synthesis to one of "reactivation" 72 h after a blood-meal (Rivera-Perez et al., 2014; Shapiro et al., 1986). The reactivation of the CA post blood feeding is an important step in the initiation of the second gonotrophic cycle; namely the initiation of previtellogenesis and the ovarian resting stage of the secondary follicles (Klowden, 1997). Thus, it is important to better understand the reproductive physiology mechanism by which CA biosynthesis is regulated and the roles that reproductive regulatory hormones like 20E might play in this process. In chapter five our findings show that in vitro stimulation with 20E at 24h
post blood feeding prematurely reactivates the gland suggesting an allotropic role for the hormone. Remarkably, stimulation with 20E resulted in an increase of JH synthesis at a time when the gland is not active. Previous metabolic analysis of the mevalonate and juvenile synthetic pathway have shown important changes in the components of these pathways that included metabolites and enzymatic activities (Rivera-Perez et al., 2014). In particular, component analysis of enzymatic activities shows that at 24 h post blood feeding the biosynthetic activity of farnesal dehydrogenase (FALDH) is not detectable, but JHAMT activity is significant. Consistent with these findings, stimulation of 20E results in an increase of FALDH activity. Just as in pupa, decapitation of 24 h blood fed females, results in premature reactivation of JH synthesis implying that 20E is mitigating the suppression of brain factors on JH synthesis after blood-feeding. Silencing of AaEcRs by RNA interference (RNAi) targeting both isoforms of the receptor for 20E did not further elucidate the role of 20E. This is not entirely surprising due to the systemic nature of silencing. In A. aegypti, EcR receptors are expressed differentially in various tissues such as the CA, midgut, salivary glands, ovary, and fat body (Wang et al., 2006; Parthasarathy and Palli, 2007). Additionally, the action of 20E via its receptors mediates many biological processes such as apoptotic responses, morphogenetic, physiological, behavioral and reproductive. Thus, it is difficult to assess how receptor silencing may be reflected on JH synthesis by the CA; particularly if the effect of 20E is a modulating one. Hence an inducible CA specific approach would yield definitive answers to the question of how 20E is capable of reactivating the CA in vivo. To
address this issue, we have begun to create GeneSwitch/UAS expression system, a bipartite system that is composed of a responder and driver line, that will allow for studies in targeted gene disruption.

Thanks to recent advances in genome editing and the guidance of Dr. Matthew DeGennaro, we have begun implementing the CRISPR/Cas9 system to create a CA specific transgenic mosquito that expresses GeneSwitch (driver), a modified GAL4 protein that is inactive until the synthetic progesterone analogue mifespristone (RU-486) is present. Using the CRISPR/Cas9 system we have integrated GeneSwitch into the JHAMT locus. JHAMT is primarily expressed in the CA, thus providing tissue specificity. Successful integration of GeneSwitch was selected using 3xP3-DsRed eye marker (Figure 3). To date, we have crossed this line with a responder line, a homozygous line containing 5UASCD8eGFP 3xP3DsRed, to begin to test the viability of the GeneSwitch protein. The developed GeneSwitch driver line could possibly be used to investigate other CA specific questions with the use of different effector lines to yield future and exciting results in the field of juvenile hormone.

6.2 Future Directions

Future directions of this study will be focused on three main aspects: 1) Elucidating the allatostatic role of the brain, 2) Defining the molecular mechanism by which 20E is mediating the activation of key JH biosynthetic enzymes, and 3) Developing of a CA specific inducible system answers to the question of how 20E can activate the CA in vivo.
The importance of circulating factors in mediating biosynthesis of prothoracic gland (PG) has been well described in the study of ecdysone and its role in molting regulation (Kopeć, 1922). However, more recently the importance of direct innervation in mediating the biosynthetic activity of the PG has been demonstrated in B. mori (Yamanaka et al., 2006; Truman, 2006). The work presented in this dissertation demonstrates a critical role of the brain as an inhibitor of JH synthesis. In both the pupa and the blood fed female, separation of the brain (denervation) from the retrocerebral complex (CA-CC) resulted in a rise of JH synthesis that was phenocopied by in vitro treatments with 20E. The nature of the in vitro denervation manipulation involves removing any regulatory innervation by the brain on the glands. Thereby it is difficult to assess if the effect of the brain on the CA is via direct innervation or through circulating factors. To better address the question possible approaches could include mapping of neuronal activity and the generation of genetic tools that could systematically block brain activity. For instance, mapping neuronal activity in the late pupa may help define plausible neuronal players which might be playing a role in the changes of JH synthesis and JHAMT activity that have been observed with 20E dynamics (Areiza et al., 2014). Likewise, the development of genetic tools such as pan-neuronal drivers could allow the targeted manipulation of neurons that could be involved in regulation of CA biosynthesis (Dolan et al., 2017). An interesting and likely scenario is the possibility that the action of the brain on the CA is a combination of both neuronal innervation and humoral factors.
One way in which metabolic networks control the flux of metabolites is through the regulation of enzymes, which can done either on a long-term or short-term scale (Oliveira et al., 2011). An example of long-term regulation is the control of gene expression, while post-translational modifications (PTMs) exemplify short-term mechanisms of enzymatic regulation. Data in this dissertation has shown that the observed effect of 20E on the activation of key JH biosynthetic enzymes is not a result of transcriptional control. As such it additional work is needed to define the molecular mechanism of 20E action of the activation of enzymatic activities of JHAMT and FALDH. A possible target would be to explore PTMS such as protein phosphorylation that could possibly modulate the enzymatic activity of JHAMT and FALDH.

Lastly, work on an inducible CA specific transgene (GeneSwitch/UAS) expression system has been initiated in this study. We have integrated GeneSwitch into the JHAMT locus using the CRISPR/Cas9 system. As mentioned in Chapter three, in the following section, a brief background on the GeneSwitch/UAS system will be discussed and it will be followed by a bulleted summary of the findings of this dissertation.

6.2.1 Bipartite systems

The ability to control gene expression is an important facet of studying gene function. The use of fusion gene constructs allows for tissue-specific control of expression; however, this method is limited by the availability of promoters and possible toxic effects from the gene product. A system that improved on these limitations is the GAL4/UAS system, developed in *D. melanogaster*, that allowed
for spatially controlled gene expression (Brand and Perrimon, 1993). Since then, the approach has been successfully implemented in several mosquito species including \textit{A. aegypti}, \textit{A. gambiae}, and \textit{A. stephensi} (Kokoza and Raikhel, 2011; Lynd and Lycett, 2012; O’Brochta \textit{et al.}, 2012; Zhao \textit{et al.}, 2014).

The GAL4/UAS system was adapted from the yeast \textit{Saccharomyces cerevisiae}, where the Gal4 gene product is responsible for activating transcription of yeast genes that encode for enzymes that convert galactose to fructose. Gal4 mediates transcriptional control by binding to an activating sequence (UAS) element that is upstream of the starting site of transcription (Duffy, 2002). The GAL4/UAS system is a bipartite approach which is comprised of two parental lines: a responder and a driver expression (Brand and Perrimon, 1993) (Figure 1). In the responder line, transcription of the target gene is under the control of a UAS element (five tandemly arrayed Gal4 binding sites) and is transcriptionally silent in the absence of the activator, Gal4 (Duffy, 2002). Conversely, in the driver line the activator protein Gal4 is expressed but without its target sequence UAS, no effects are seen. In order to activate transcription of the gene of interest, both lines have to be crossed. Their resulting progeny will express the gene under UAS control in cells where Gal4 is also expressed. In this manner, the GAL4/UAS system allows for targeted gene expression that is cell or tissue specific.

An additional level of temporal and spatial control of UAS-transgene expression was achieved through the GeneSwitch/UAS expression system developed in \textit{D. melanogaster} (Osterwalder \textit{et al.}, 2001). In this approach,
Inducible gene expression is achieved by controlling both the expression of Gal4 proteins through tissue-specific promoters and its transcriptional activity in the presence of a ligand. The Gal4 GeneSwitch protein is the product of chimeric Gal4 gene that encodes a ligand-binding domain for the activator, RU486 (mifepristone) and a Gal4 DNA-binding domain. Like the GAL4/UAS system, the GeneSwitch/UAS expression system is a bipartite approach (Figure 2). The driver line expresses the activator, Gal4 GeneSwitch, in a tissue specific fashion. While the responder line, transcription of the target gene is silent in the absence of the activator. Once both lines are crossed, transcription of the target gene is activated only in the presence of RU486. This steroid ligand elicits a conformational change in the Gal4 GeneSwitch protein and then becomes transcriptionally active, driving expression of the target gene. Aside from the inducible and tissue specific control of gene expression that this system provides, there is the added benefit of being able to control the transgene expression levels in a dose dependent manner by varying the dosage of RU486 (Osterwalder et al., 2001).

In summary, the ability to control gene expression temporally and spatially has greatly advanced molecular genetic studies. Targeted gene expression was first introduced by the GAL4/UAS system which allowed for the study of gene function with cell type specificity. But it was the GeneSwitch/UAS system that has introduced a greater control of gene expression by creating an inducible and tissue-specific transgene expression system using the inducible Gal4 protein, GeneSwitch.
6.3 Summary

Taken together, the observations presented in this dissertation demonstrate that 20E plays an allotropic role in CA activity via the activation of key JH biosynthetic enzymes. Also, it suggests that both in the pharate adult (late pupal stage) and in the blood fed female, the brain suppresses JH biosynthesis and 20E was able to overcome this inhibition. More work needs to be done to better determine how 20E is mediating the activation of the enzymatic activity of both JHAMT and FALDH respectively and what brain factor is suppressing JH synthesis in the CA. The finding in chapter 4 and 5 include:

- 20E acts overrides the inhibitory action of the brain.
- Separation of the brain from the CA “wakes-up” the CA.
- In -24h pupa \textit{in vitro} treatments of 20E initiate JH biosynthesis.
- Application of 20E correlated with an increase in the enzymatic activity of juvenile hormone acid methyltransferase (JHAMT) at -24h.
- At 24h post blood feeding, \textit{in vitro} stimulation with 20E prematurely reactivates the CA.
- Treatment with 20E increases the activity of FALDH 20E at 24h post blood feeding.
- Silencing of AaEcRs by RNA interference (RNAi) did not reflect changes in JH levels.
- Integration of GENESWITCH into the JHAMT locus using CRISPR/CAS9 system and preliminary testing of the line.
Figure 1. Directed gene expression using the GAL4/UAS binary expression control system. In the responder line, the target gene is under the control of a UAS element and in the absence of GAL4 it remains transcriptionally absent. When the two lines are crossed, Gal4 protein is expressed and binds to UAS activating the gene in a tissue specific manner.
Figure 2. Generation of two mosquito using the GeneSwitch/UAS system. The driver line expresses the activator, Gal4 GeneSwitch under a tissue specific promoter. The responder line, UAS line, contains the target gene which is driven by UAS (GAL-4 binding sites). Both lines are crossed, the activator (RU-486), elicits a conformational change in the GeneSwitch protein, it becomes transcriptionally active.
Integration of GENESWITCH into JHAMT locus using CRISPR/CAS9 system

![Diagram showing integration process]

**Figure 3. Integration of GeneSwitch into JHAMT locus.** Using the CRISPR/Cas9 system a region containing GeneSwitch and 3xp3 DsRed eye marker will be inserted into the JHAMT locus for screening of mutants.
References


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