A Novel Function for Juvenile Hormone in Male Courtship Behavior of *Drosophila melanogaster*

A Dissertation

Presented to the

Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment of the

Requirements for the Degree

Doctor of Philosophy (PhD)

By

Thilini Priyadharshi Wijesekera

August 2013

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Acknowledgements

There are many people that I wish to thank at the stage of completing this project and this dissertation. They have helped me in many ways to reach this significant goal in my life.

First and foremost, I wish to extend my heartfelt gratitude to my PhD advisor, Dr. Brigitte Dauwalder, for being an excellent and inspiring scientist and for all her guidance on my research and dissertation. I am grateful to her for giving me the opportunity to work in her lab and for giving me this project from which I have learned a great deal. I thank her for her guidance in all aspects of my graduate student life and the PhD program during these five years. Working in her lab has been an enjoyable and enriching experience. I thank her for all her kindness during my time as a graduate student in her lab and her advice and encouragement during the many challenging times of this project.

I have been very fortunate to have worked closely with a very experienced and dedicated group of scientists, Dr. Gregg Roman, Dr. William Mattox, and Dr. Cecilia Williams, who comprised the remaining members of my dissertation committee. Their expert help and guidance have served to bring this project to the standard it achieved through these years. I thank them for always being there when I needed their help and advice and for working together as a team for the collective benefit of this project. Working with them has been a wonderful and rewarding experience.

I wish to thank Chamala Lama, Valbona Hoxha, Yuanli Li, and Nancy Vanaphan for being the best lab members anyone could hope for. In this lab I found a truly unforgettable group of friends. I thank them for all the companionship, memorable times, and support during the hard times of

grad student life. In them I found a group of surrogate sisters who made me feel loved and appreciated.

Many thanks to Ms. Amanda Paul for all her help with the administrative side of my graduate student life. I thank her for all the times she helped me and watched over me, so I could focus on what matters to achieve my goals. I thank her for being such a wonderful human being; the world definitely needs more people like her. I also thank Ms. Rosezelia Jackson for all her help on administrative issues.

I wish to thank my friends from Dr. Amy Sater's lab; Yi-Er Shieh, Ruth Ritter, and Vrutant Shah for all their help with the various experiments and also for being such a great bunch of friends. My friends in Dr. Gregg Roman's lab; Adrian Madalan, Emmiliano Fumangalii, Claire Mason-Bishop, Shiyu Xu, and Shixing Xang for all their help with experiments and all their friendship.

I wish to thank Sirinivas Patnaik and Sudeep George of Dr. Seema Khurana's lab for all their expert help on the confocal imaging.

Collectively, I have been very fortunate to have worked with such a culturally diverse group of people, which also served to enhance my experience of grad student life. It has been a great five years. I could not have hoped for more.

I wish to extend my deepest gratitude to my uncle and aunt, Drs. Gemunu and Preethi Herath Guneratne, for inspiring me to be a scientist at a very young age, for all their help and guidance at every step of the way and for believing that I can and will achieve this goal.

I thank my sister and brother-in-law, Radhika and Dharshan Gunawardena, for all their help and support, and baby Maneka for putting a smile on my face.

Lastly and very importantly, I would like to take this opportunity to extend my deepest heartfelt gratitude to my parents, Dr. S.G. Wijesekera and Mrs. P. Wijesekera. I thank them for raising me to be the person I am. I thank them for all their sacrifices to see me reach this goal and for their strength and support through every stage of my life. They inspired me to believe in myself, to follow my own passions and be the best I can be.

To my parents, I dedicate this dissertation.

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ABSTRACT

Juvenile hormone is a significant insect hormone controlling development as well as reproduction, migration, and social behavior. Juvenile hormone-binding proteins of many insects are similar to *Drosophila melanogaster* 'Takeout', a protein preferentially expressed males that influences courtship behavior. This raises the possibility for a role for Juvenile hormone in male courtship behavior. This hypothesis was tested by creating flies with reduced Juvenile hormone levels and examining their mating behavior. To achieve a reduction in levels, a key enzyme of the Juvenile hormone synthesis pathway was targeted by RNA interference (RNAi). Juvenile Hormone Acid Methyl Transferase (*JHAMT*) catalyzes one of the last steps in the hormone's synthesis in the Corpora allata, the organ of Juvenile hormone synthesis. A new Corpora allata-specific GAL4 driver line was created and used to direct *JHAMT*-RNAi to these cells. Courtship assays of the resulting males showed that RNAi against JHAMT results in a mutant courtship phenotype. Constitutive reduction as well as specific adult reduction reduced courtship. The same result was also obtained by conditional genetic ablation of the Corpora allata in adults. The courtship defects could be rescued by application of the Juvenile hormone analog Methoprene shortly before behavioral testing. Together, these results show that normal adult Juvenile hormone levels are physiologically required for normal male courtship behavior.

It is unknown which protein(s) transport Juvenile hormone in *Drosophila melanogaster*. Since Takeout has many of the characteristics of JHBPs, we tested whether Takeout binds the hormone to act as a carrier for the hormone. No binding was observed between a Baculovirus produced Takeout protein and Juvenile hormone in an *in vitro* binding assay.

The findings presented in this dissertation demonstrate a novel and important function for Juvenile hormone in the control of male courtship behavior in *Drosophila melanogaster*.

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CHAPTER I

INTRODUCTION

CHAPTER I: INTRODUCTION

1.1 Courtship behavior in *Drosophila melanogaster*

Courtship behavior in flies is well studied and characterized. The genetics and neural circuitry involved in the behavior is a fascinating area of research and has given rise to very significant findings related to innate behavior. It is robust and elaborate and is composed of steps that are followed to an exact order. The male fly is capable of performing the behavior even if he is raised in isolation, upon contact with a female as his first encounter with another creature, indicating that it is an innate behavior. The male engages in a series of actions; briefly, orienting towards the female, following, tapping her abdomen with his forelegs, wing extension towards her and performing a species specific courtship song, licking her genitalia and attempting copulation (Greenspan and Ferveur, 2000; Hall, 1994). All of the above steps are performed in order. If the targeted female is a mature virgin and she is receptive to mating, copulation takes place (copulation is not a component of the courtship behavior). If the female is an immature virgin or is a newly mated female, copulation does not take place as she is not receptive. Receptivity by the female is indicated by her slowing down and opening her ovipositor in an attempt to copulate. Copulation takes place for 20 minutes. Spieth (Spieth, 1974) suggests that each of the sequential steps of the behavior sets a threshold levels that leads into the next step.

The courtship behavior in flies can be analyzed relatively easily in the laboratory and is very quantitative. It is well suited for genetic analysis. The details of the various components of the behavior are as follows (Figure 1) -

1. Orientation- The behavior initiates with the male orienting towards the female. The time period to first orient to the female is known as the courtship latency and is dependent on visual, auditory, and pheromonal stimuli. Following orientation, the male will proceed to follow the female in an attempt to make contact. Usually any female, receptive or un-receptive, will initially move away from the male.
2. Tapping- The initial physical contact a courting male makes with a female and is driven by pheromonal cues on the female's body. The male taps the abdomen of the female with his forelegs. The forelegs have been shown to contain gustatory receptors that detect the pheromones; Gr32a, Gr33a, and Gr68. They are involved in the detection of species specific pheromones on the female by which the male identifies her as one of his own species.
3. Singing- The male performs a species specific courtship song by extending the wing closest to the female and vibrating it to produce a rhythm. This courtship song is composed of 'sine' and 'pulse' components and an inter-pulse interval (IPI). The song stimulates the female to mate, while providing her with information that he is of her own species.
4. Licking of genitalia- The male licks the female's genitalia with his proboscis
5. Attempted copulation- the male curls his abdomen and contacts the tip of his abdomen with that of the female.

Several stimuli are instrumental in initiating and maintaining this behavior (Figure 1). These stimuli, visual, gustatory, and acoustic stimuli are means of communication between the two partners during the course of the behavior and they function to activate the relevant neural

circuits pertaining to the various steps. Visual stimuli are both dynamic (locomotor activity, wing displays, and motion) and static (colors and shapes) (Greenspan and Ferveur, 2000). The female locomotor activity is an important first determinate for a male to initiate courtship. It facilitates recognition of the female as a conspecific (Tompkins et al., 1982).

Acoustic signals are the stimuli that a male passes to a female in the form of wing vibration and courtship song. Females receive the auditory stimuli by means of their feathery antennae (Cook, R.M., 1979)and are stimulated to be more receptive (von Schilcher, F., 1976).

Gustatory signals are present in the form of pheromones. It is believed that a majority of chemical substances that act during courtship are detected by gustation during tapping and licking. Stimulatory pheromones are gustatory (Greenspan and Ferveur, 2000).

The female undergoes a series of behavioral changes after mating, including loss of receptivity, increased egg-laying, and synthesis of pheromones that are inhibitory to males (Manning, 1967). Chemicals and peptides synthesized by the accessory gland of males are transferred to females in the seminal fluid and are instrumental in causing these changes (Chen, P.S., 1991; Wolfner, 1997).

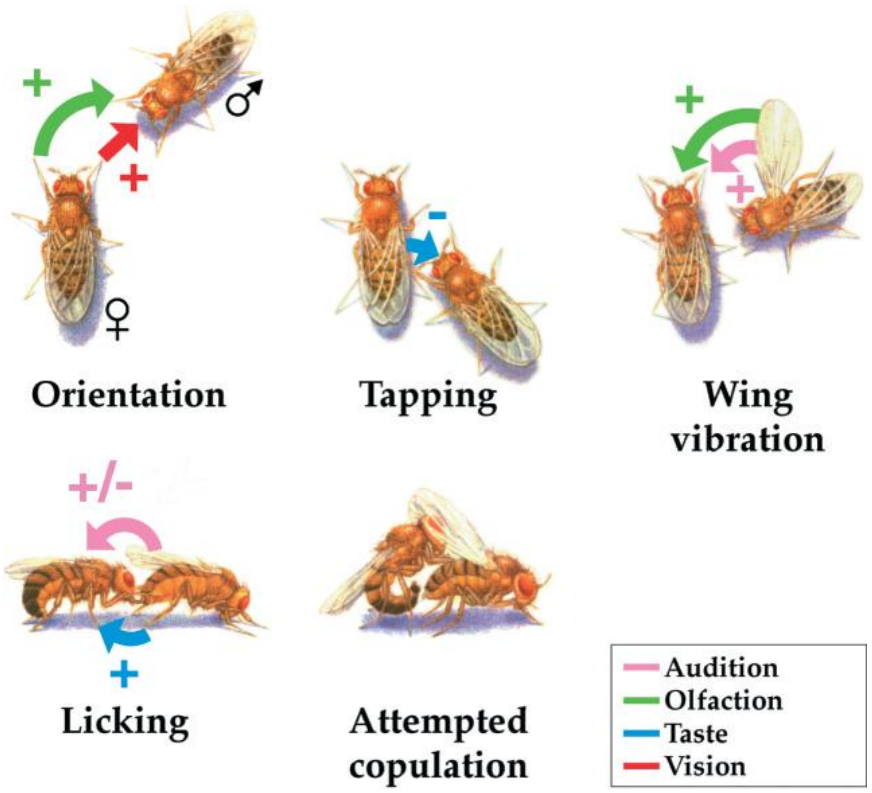


Figure 1: The sequence of behaviors in heterosexual courtship of *Drosophila melanogaster*. The colored arrows represent sensory modalities by which flies communicate: (+) for stimulatory. (-) for inhibitory (Greenspan and Ferveur, 2000).

1.1.1. Sex determination and dosage compensation in *Drosophila*

Male courtship behavior is regulated by the same mechanisms of sex determination that determine overall sexual differentiation. The choice of sexual cell fate is a developmental decision. An initial signal is transmitted through a regulatory cascaded to activate genes required to produce the distinct features of a particular sex (Figure 2). This initial signal varies in

different species. Vertebrates use a male determining factor on the Y chromosome (Ford et al., 1959; Jacobs and Strong, 1959; Welshons and Russell, 1959). *Drosophila melanogaster* and *Caenorhabditis elegans* use the X chromosome to Autosome (A) ratio (Cline and Meyer, 1996).

Sex determination in *Drosophila* was shown to be generally cell autonomous; most cells deciding their own sex (Baker and Ridge, 1980). The regulatory genes in males and females are different due to the differential splicing of their RNA transcripts and those of their regulatory genes upstream in the cascade. Therefore, sex determination in *Drosophila* is due to a cascade of sex-specific alternative RNA splicing that determines whether the cell is male or female (Baker, 1989).

The primary determinant in *Drosophila* is the ratio of X to A (autosomes) (X:A) (Bridges, 1921). When the ratio is 1:2 the flies or cells are male, and when 2:2 they are female. The Y chromosome does not function in sex determination. The X:A ratio of 2:2 (female) activates the *Sex lethal* (*Sxl*) gene (Cline, 1984; Cline, 1978). The 'default state' in *Drosophila* is the 1:2 (male), where the gene *Sxl* gene does not have a function (Salz et al., 1987).

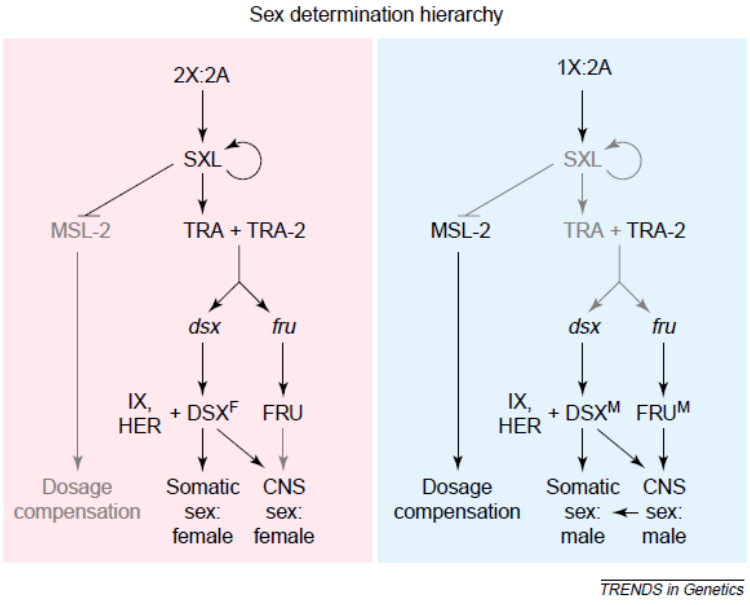
The Sxl protein functions in two ways; it activates its own splicing, thereby creating a memory of its sex in the cell (Cline, 1984), and controls expression of downstream regulatory genes that control somatic sexual differentiation, germline sex determination, and dosage compensation (Baker, 1989) (Figure 2).

Sxl is a splicing factor activated by maternally and zygotically provided gene products, the maternally provided *daughterless* (*da*) and zygotically provided *sis-b*, *sis-a* and *snf* (Baker, 1989; Cline, 1978, 1980, 1988; Oliver et al., 1988). Sxl protein regulates somatic sexual differentiation in females through the control of the gene *transformer* (*tra*). Its gene product functions only

when activated by Sxl. i.e. in females and is regulated at RNA splicing (Baker and Ridge, 1980; Boggs et al., 1987; McKeown et al., 1988). In the male 'default state', the absence of *Sxl* causes the first exon of the *tra* pre-mRNA to be spliced at a male specific splice site, resulting in an inactive Tra protein. In the female state, expression of *Sxl* causes the splicing to occur at a female specific splice site to produce an active Tra protein. The *tra* product collaborates with *tra-2* (constitutively expressed in males and females (Amrein et al., 1988; Goralski et al., 1989)) to control the splicing of *doublesex* (*dsx*) and *fruitless (fru)* RNAs (Nagoshi et al., 1988). The Tra protein expressed in females acts with the Tra-2 protein to cause the female specific splicing of *dsx* pre-mRNA to produce dsx^F mRNA (Hoshijima et al., 1991). When *Sxl* is absent (the male state), the absence of functional Tra causes the male specific splicing of *dsx* pre-mRNA to produce dsx^M mRNA (Figure 2). The male and female specific *dsx* transcripts were both shown to encode active Zinc-finger transcription factors, Dsx^M and Dsx^F in males and females respectively (Hoshijima et al., 1991; Ryner and Baker, 1991). Tra and Tra-2 in females were also shown to sex-specifically splice the *fru* pre-mRNA to produce a female fru^F transcript. This female fru^F transcript does not produce a functional protein. The male specific fru^M transcript is translated to produce a BTB-zinc finger transcription factor that is responsible for the sexual differentiation of male features and some male sex-specific behavior (Christiansen et al., 2002).

Therefore in *Drosophila*, male specific splicing ("housekeeping" splicing patterns) occurs in the absence of female specific gene products, producing the 'default' male state, while the female state is the consequence of Sxl, Tra, and Tra-2 activity. These genes modify the housekeeping machinery to form the female specific mRNAs of *Sxl*, *tra* and *dsx*.

In females, *dsx*^{F} acts with *intersex* (*ix*), to repress the genes involved in terminal differentiation specific to males. The genes acting to cause female differentiation are not repressed and therefore, female differentiation occurs. The inverse occurs in males.



Christiansen et al., 2002

Figure 2: The regulatory genes controlling the somatic sexual differentiation in *Drosophila* (Christiansen et al., 2002).

1.1.2. Sex determination pathway controls male sexual behavior in *Drosophila*

Sex-specific behaviors are dependent on a sexually dimorphic nervous system (Baker et al., 2001; Billeter et al., 2002) and the dimorphism in the nervous system was shown to be

determined by the sex determination pathway (Cline and Meyer, 1996; Christiansen et al., 2002). Initially it was believed that, male sexual behavior is determined entirely by fru^M, while somatic sexual differentiation outside the central nervous system (CNS) is controlled by dsx^M (Billeter et al., 2002; Baker et al., 2001). However, studies have shown that *dsx* mutant XY flies show reduced courtship and that their courtship song is defective (Villella and Hall, 1996; Pan et al., 2011; Rideout et al., 2007; Shirangi et al., 2006).

fru was long believed to be the only regulatory gene determining courtship behavior (Ito et al., 1996; Ryner et al., 1996). It has been shown to be expressed in defined regions of the Central Nervous System (CNS) (Baker et al., 2001). *fru* mutations affect nearly all the steps of the courtship behavior (Goodwin et al., 2000; Ryner et al., 1996; Villella et al., 1997). *fru* null mutants do not court, and hypomorphic mutants were shown to be unable to differentiate between the sexes and to attempt courtship on males and females equally. Furthermore, they were shown to not produce a courtship 'pulse' song, although they do rarely extend their wing towards the female (Goodwin et al., 2000; Ryner et al., 1996; Villella et al., 1997). It was also found that *fru* is required for seminal fluid and sperm transfer and for a normal duration of mating (Lee et al., 2001).

Wildtype *dsx* function is required in both sexes for somatic sexual differentiation (Christiansen et al., 2002; Cline and Meyer, 1996). Furthermore, dsx^M was shown to be required for the normal execution of courtship behavior (Pan et al. 2011; Rideout et al., 2007; Shirangi et al., 2006; Villella and Hall, 1996). However, *dsx* is insufficient to control courtship behavior on its own as flies expressing only dsx^M are male in appearance, but do not court females (Taylor et al., 1994). *dsx* was also shown to be expressed in the CNS in flies (Lee et al., 2002).

1.1.3. The f*ruitless* (*fru*) and *doublesex* (*dsx*) genes in courtship behavior and its neuronal circuitry

The neural circuitry responsible for the execution of courtship in *Drosophila* is extensively being studied, mainly based on the expression of the 'master regulator' genes of courtship behavior, *fru*, and *dsx*. Since Fru and Dsx are sex specifically expressed in males and females, it was hypothesized that they function in the development of a sexually structurally dimorphic neural circuitry of courtship.

The regulation of male courtship in *Drosophila melanogaster* was shown to be dependent on the function of *fru* to a large part (Baker et al., 2001; Billeter et al., 2002; Demir and Dickson, 2005; Manoli et al., 2005). *fru* has two characterized functions; controlling male sexual behavior (Billeter et al., 2006b; Demir and Dickson, 2005; Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996) and viability in both sexes (Billeter et al., 2006b). The gene encodes a group of transcriptional regulators, each containing a BTB protein-protein interaction domain and a zinc finger. Molecular cloning of the *fru* gene showed that the transcripts produced include both sex specific and non-sex specific transcripts. The *fru* gene produces four transcripts, one of which is driven by the P1 promoter (the most distal) and is sex specifically spliced in males and females, and the others, driven by P2, P3, and P4 promoters are common to both sexes. The male specific P1 transcript produces the Fru^{M} protein (Baker et al., 2001; Ryner et al., 1996). This sex-specific transcript is spliced in a Tra and Tra-2 dependent manner (Ryner et al., 1996).

Mutations in the *fru* gene have been shown to be associated with significant reduction or absence of courtship towards females, defective courtship song, increased male-male courtship and failure to copulate (Billeter et al., 2006a). The *fru* mutants that do copulate were shown to

be defective in seminal fluid transfer and are sterile (Lee et al., 2001). When *fru* was expressed in females, the females could perform the early steps of courtship, but they rarely performed licking of genitalia and were unable to attempt copulation (Demir and Dickson, 2005; Manoli et al., 2005). Therefore, apparently *fru* alone cannot execute the complete normal courtship behavior in flies; further male specific factors also seem required.

Another gene hypothesized to have a role in courtship behavior was *dsx*. The gene *dsx* is also regulated by the sex determination pathway at the level of splicing by the same splicing factors, *tra* and *tra2* (Hoshijima et al., 1991; Ryner and Baker, 1991). Additionally, *dsx* mutations affect sex determination. *Dsx* proteins are part of the Dmrt (*doublesex* and mab-3-related transcription factor) which are structurally and functionally conserved zinc-finger transcription factors (Zarkower, 2002). Previous research has shown a role for *dsx* in somatic sexual differentiation outside of the CNS in both sexes (Christiansen et al., 2002; Cline and Meyer, 1996). It is insufficient on its own to control sexual behavior in males, as otherwise normal females expressing DSX^M appear male but do not display courtship behavior (Taylor et al., 1994). Males lacking *dsx* have intersexual morphology and perform courtship at diminished levels and do not perform the sine component of the courtship song (Villella and Hall, 1996). Dsx^M was seen to be required for the performance of a normal courtship song containing both 'sine' and 'pulse' components (Rideout et al., 2007).

Therefore, it is apparent that dsx^M and fru^M both contribute to courtship behavior, and this idea is strengthened by the fact that the two genes are co-expressed in many neurons (Billeter et al., 2006b; Rideout et al., 2007). Kimura et al., (2008) have identified a male specific neuronal cluster (P1) in the fly brain that co-expressed the two genes. This P1 region of the brain was

shown by the above authors to be located dorsal posterior to the mushroom body and to be composed of 20 interneurons. The authors showed that these neurons are involved in the initiation of courtship behavior.

The collaborative action of fru^M and dsx^M is required for the differentiation of the brain in the pre-adult stages of the life cycle. Lee and colleagues (Lee et al., 2000) have shown that the FRU^M protein is expressed in about 2000 neurons and that expression increases during the pupal stage and persists well into adulthood. Dsx^M is expressed in the 3rd instar larvae, pupae, and adults. Further experiments confirmed its expression in neurons (Lee et al., 2002). The peak expression is observed during the period of male programming of the CNS that occurs in the late pupal stage (Belote and Baker, 1987; Billeter et al., 2006a).

The *dsx* gene was shown to control courtship behavior in *Drosophila* (Rideout et al.,2010). Differences were observed between the *dsx* positive neurons in the fly brain regarding the axonal numbers, projections, and synaptic density. The study shows the requirement of dsx^M for the development of male specific neurons that co-express *fru* and that *dsx* neuronal function is essential for normal courtship behavior.

Studies carried out by Manoli and colleagues (Manoli et al., 2005) and Stockinger and colleagues (Stockinger et al., 2005) to explore the neural substrates defined by *fru*, used homologous recombination to insert a GAL4 gene into the P1 *fru* promoter (the promoter driving the sex specific transcript), creating a functionally null P1 *fru* called *fruP1-GAL4*. Because the *fruP1-GAL4* transcript was common to both sexes, the authors could examine for sex-specific differences in the features of neurons expressing the transcript. They discovered that the principal features of the projections of these neurons are very similar between the two sexes. The study suggests

that Fru^M does not specify the major neuronal structure in the development of these neurons, but is mostly involved in their fine connectivity and/or physiology. Therefore, it is likely that the neurons function differently because of the presence of Fru^M in males and its absence in females. Upon transient inactivation of these neurons using a temperature sensitive shi^{TS} allele, they showed that the Fru^M expressing neurons are dedicated to courtship. Several studies (Lee et al., 2002; Rideout et al., 2007; Sanders and Arbeitman, 2008) have demonstrated that sexual dimorphism in cell number of Fru^M neurons is dependent on both *fru* and *dsx.* The *fru* neurons and their potential connections in male and female brains have been extensively studied by (Cachero et al.,2010; Yu et al., 2010). Cachero and colleagues (Cachero et al.,2010) carried out the first voxelwise analysis of structural differences in an insect brain. They detected extensive sex-specific volume differences between males and females. Their study shows that one third of the *fru* neurons are dimorphic between sexes. Most dimorphisms were seen to be in the arbors of *fru* higher interneurons of the protocerebrum. The increased density of these neurons in males, when compared to females, could function to increase connection strength between neurons. A study carried out by Yu and colleagues (Yu et al.,2010) used genetic methods to dissect the *fru* neurons into distinct subsets and compiled a digital 3D atlas at cellular resolution. They arranged these neurons into circuits extending from sensory input to motor output. Eleven anatomical dimorphisms were detected: Neurons that are male specific and neurons that are more numerous and have distinct arborizations in males, when compared to females. Yu et al. described a core of the *fru* circuit in the brain that is strongly connected to the dorsal medial protocerebrum and the mushroom body and the lateral protocerebral complex. The region, while being enriched in *fru* neurons, is believed to be where integration of sensory inputs and motor outputs occur.

1.1.4. Downstream effectors of *fru* and *dsx* and the role of the fat body

The functions of *fru* and *dsx* in courtship behavior are well established through a body of complex and extensive research studies in *Drosophila* as referenced above. However, the downstream genes they regulate have not been explored as intensively. Several downstream genes regulated by *fru* and *dsx* have been identified by several screens (Arbeitman et al., 2004; Dauwalder et al., 2002; Fujii and Amrein, 2002; Goldman and Arbeitman, 2007). The best studied DSX downstream genes are the female *yolk protein* (*yp*) genes which are directly bound and regulated by DSX^F. In this context, Dsx^F protein appears to be a direct activator while Dsx^M protein appears to be a direct repressor. Although the male preferentially expressed *takeout* gene is expressed at normal wildtype levels only under the normal levels of Fru^M and Dsx^M (Dauwalder et al., 2002), direct binding of the transcription factors to the *takeout* gene has not been demonstrated.

However, previous research has indicated a functional role in courtship behavior in *Drosophila* for the fat body (Lazareva et al., 2007), and *takeout*, a gene confirmed to have a role in male courtship behavior, is expressed in the head associated fat body of males (Dauwalder et al., 2002). Therefore, the fat body appears to be a non-neuronal tissue involved in male courtship behavior. The fat body (adipose tissue) is a major secretory tissue in larvae and adults. It is composed of large lipid-filled cells and has a crucial role in fat storage, energy metabolism, and immunity (Bownes and Hames, 1977; Haunerland, 1996; Meister et al., 1997). It secretes factors into the hemolymph of the fly. In females, the organ appears to have a sex-specific function in the production of a large amount of yolk proteins (Bownes and Hames, 1977). The cells attain

their maximum size within one week of eclosion and function as a storage tissue accumulating lipid and glycogen (Butterworth and Bodenstein, 1968).

Lazarava and colleagues (Lazareva et al., 2007) demonstrated that a male-specific fat body is required for normal courtship in addition to Fru^M. When Fru^M is expressed in females, they court other females with a reduced courtship index. However, when the whole fat body is masculinized in addition, a complete normal courtship index was achieved. Also, when the fat body of normal males was feminized using specific drivers driving expression of Tra, their courtship decreased significantly. Therefore, the sexual identity of the fat body appears to be essential for normal courtship. These observations suggest a communication between the fat body and the courtship neural circuits controlling courtship behavior in *Drosophila*.

Several studies have identified *fru* and *dsx* target genes that are expressed in the fat body (Ellis and Carney,2010; Ellis and Carney,2011). The best characterized of them are *takeout*, *fit*, *sx1* and *sx2* (Dauwalder et al., 2002; Fujii and Amrein, 2002). A function for male courtship behavior has been established for *takeout*, which is expressed in the head fat body male preferentially and secreted into the hemolymph (Dauwalder et al., 2002), which indicates an important role for secreted circulating molecules in this behavior. An unanswered question is how *takeout* and other factors that are expressed and secreted into the hemolymph communicate with the neuronal circuits to execute this behavior. The *takeout* protein contains features of a soluble carrier protein of lipophilic molecules. A property it shares with Odorant Binding Proteins (OBPs). It shows high similarities to JHBPs of Lepidopterans. Binding between Takeout and Juvenile hormone has not yet been demonstrated. However, a courtship role for Juvenile hormone has been suggested in studies of mutants with alteration of Juvenile hormone levels

and altered Juvenile hormone signaling. *apterus* (*ap*) and *Methoprene-tolerant* (*Met*) mutants show some courtship defects (Ringo et al., 1992; Wilson et al., 2003). Apterus (*ap*) mutants were shown to have reduced Juvenile hormone levels (Altaratz et al., 1991) and Juvenile hormone signaling is known to occur through Met (Riddiford, 2012).

1.2. *Takeout* (*to*) and Juvenile Hormone Binding Proteins (JHBPs)

Takeout is a *Drosophila* gene that was independently identified in two different subtractive hybridization screens: One for clock-regulated circadian output genes, and one for sex-regulated genes (Figure 3). It was found to encode a circadian cycling transcript whose transcript was undetectable in cyc^{01} and Clk^{jrk} mutants (So et al., 2000), although this finding was later shown to be due to the presence of two different *takeout* alleles in these strains (Benito et al., 2010). Initial functional analysis of *takeout* by Sarov-Blat and colleagues (Sarov-Blat et al., 2000) suggested that it is involved in feeding behavior in response to starvation. The protein was found to have similarities to JHBPs, such as *Msh*JHBP and *Hvh*JHBP (Figure 4 (B)). Takeout and hemolymph JHBP from *Manduca sexta* show a 24% identity and 54% similarity with hemolymph JHBP of *Manduca sexta* (Sarov-Blat et al., 2000). Of particular interest is the sequence similarity in the N-terminal ligand binding region of JHBPs. Studies by Wojtasek and Prestwich (Wojtasek and Prestwich, 1995) have shown that an N-terminal pair of Cystine (Cys) residues is required for Juvenile hormone binding in JHBPs. These Cys residues are absolutely conserved within the whole *takeout* family. This suggests that Takeout has ligand binding property and could be a JHBP (Sarov-Blat et al., 2000). The Takeout protein has an N-terminal predicted secretion signal,

and Lazarava and colleagues (Lazareva et al., 2007) have shown that Takeout is present in the hemolymph.

There are 24 *takeout*-related genes in *D. melanogaster* (Figure 4 A). Takeout homologues have been identified in several other insects in a variety of tissues (Du et al., 2003; Fujikawa et al., 2006; Ghanim et al., 2006; Hagai et al., 2007; Hamiaux et al., 2009; Jordan et al., 2008; Saito et al., 2006; Schwinghammer et al., 2011) A recent phylogenetic analysis has shown that the gene family is old and conserved in insects (Vanaphan et al., 2012). No homologues are present in vertebrates.

(A)

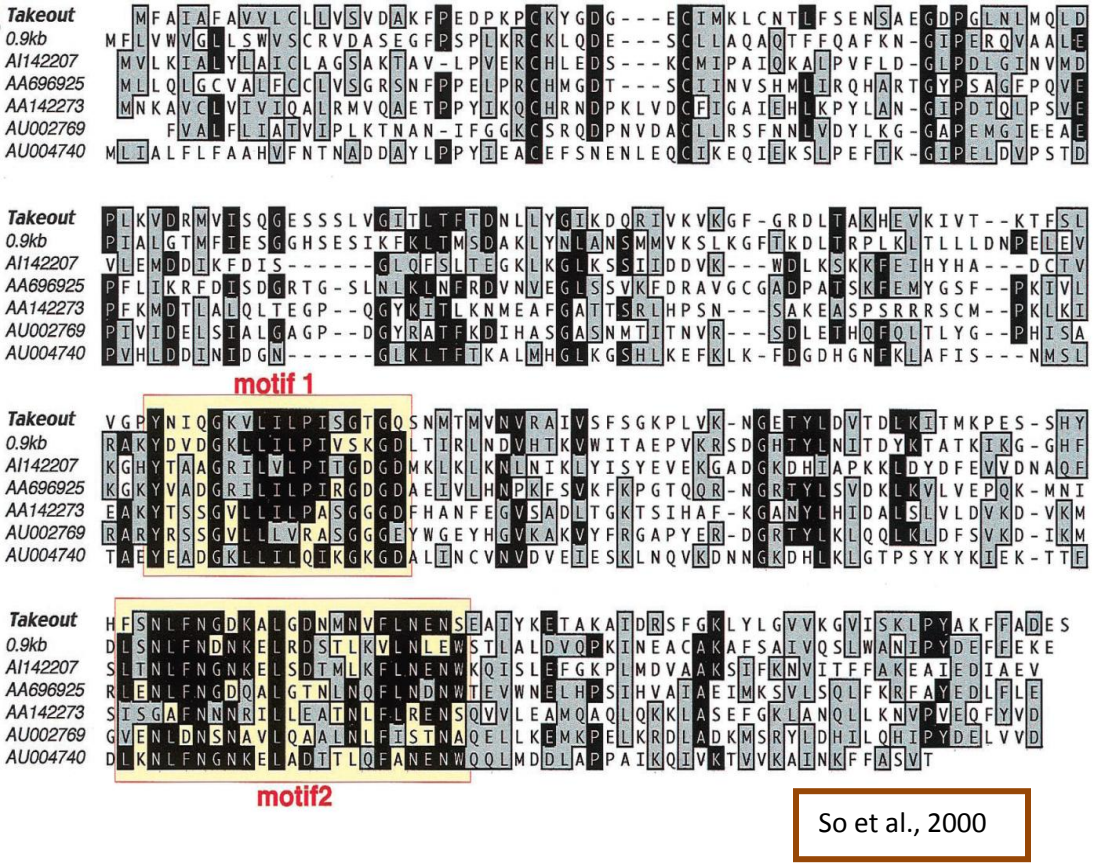
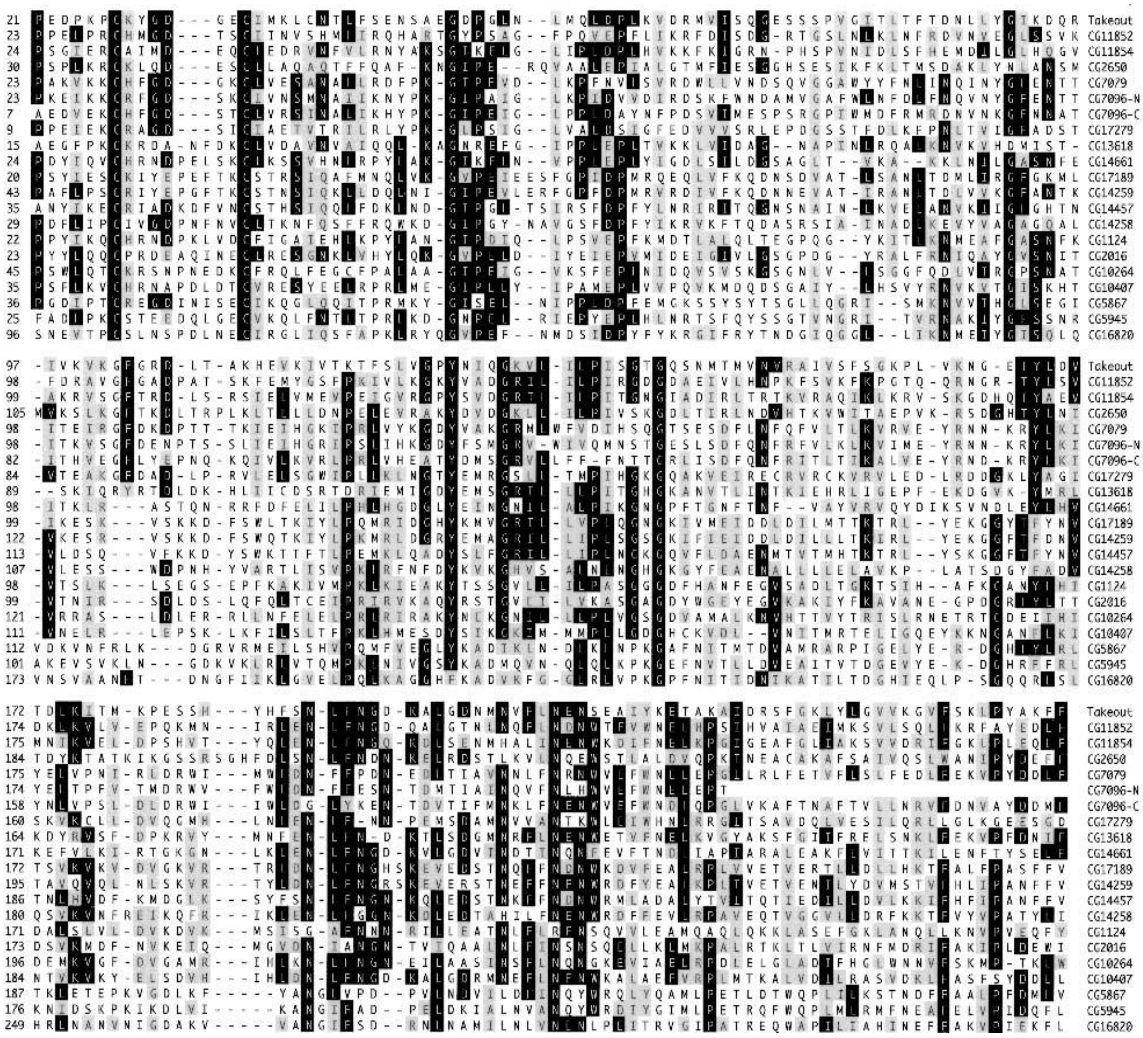


Figure 3: Takeout protein sequence alignments I. Alignment of *takeout* family members. Boxes indicate signature motifs that define the *takeout* family. The family members from *Drosophila melanogaster* shown are *takeout*, 0.9kb, AI142207, AA696925, and AA142273 (So et al., 2000).

(A)



Sarov-Blat et al., 2000

(B)

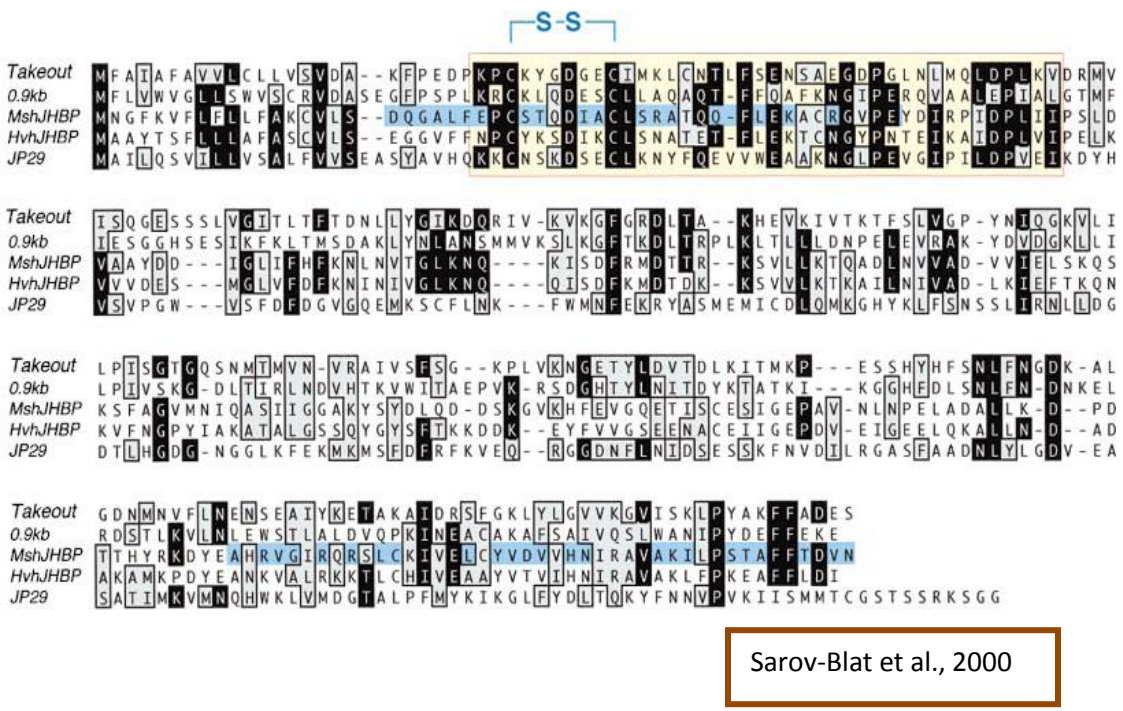


Figure 4: Takeout protein sequence alignments II. (A) Sequence alignment of the 20 members of the *takeout* family of *Drosophila* (Dauwalder et al., 2002). (B) Sequence alignment of the *takeout* family with the JHBPs. The hormone-binding fragments are highlighted in blue. The disulfide bond is indicated. The box indicates the region of highest identity between *takeout* and the ligand-binding proteins (Sarov-Blat et al., 2000).

Takeout has also been identified in a screen for sex-specific genes in fly heads that are expressed under the control of the sex-determination gene *tra-2*. The expression of *takeout* is male specific, with high expression in heads. The *takeout* gene is expressed male-specifically in brain- associated fat body, and not sex-specifically in the antennae (Dauwalder et al., 2002). The brain associated expression, which is sex-specific was seen to be regulated by *transformer-2*. The antennal derived takeout is only a small percentage of the total *takeout* expressed in the fly. The above study also demonstrates that *takeout* expression depends on the presence of male specific isoforms of Dsx^{M} and Fruitless Fru^{M}. In addition to *takeout*, several other *D. melanogaster* homologues have also been shown to have male-biased expression (Dauwalder et al., 2002; Vanaphan et al.,2012).

A function of *takeout* in male courtship behavior has been identified by Dauwalder and colleagues (Dauwalder et al., 2002). The *takeout* mutants have courtship defects. That the sex-specific identity of *takeout* expressing cells is important for courtship has been shown by feminization of these cells in otherwise normal males. The feminization of *takeout* expressing cells using *UAS-tra*^{F} driven by *takeout-GAL4* resulted in a large reduction in courtship index (Dauwalder et al., 2002). Feminization using a fat body specific GAL4 driver confirmed the importance of male-specific molecules in the fat body (Lazareva et al., 2007). The courtship mutant phenotype obtained by feminizing the adult male fat body is significantly stronger than that obtained by a specific *takeout* mutation alone as done by Dauwalder and colleagues (Dauwalder et al., 2002). This argues to the fact that there are additional factors expressed in the adult male fat body that are required for normal male courtship. Indeed, several authors have identified other male-specifically expressed transcripts in the fat body. However, there are few functional studies on their role to date. The *takeout* homozygous mutants were shown to

have a courtship defect. The *takeout* gene interacts genetically with a mutation in *fru*, demonstrating that it acts in the overall pathway required for male courtship (Dauwalder et al., 2002).

Several studies have further indicated a function for *takeout* in feeding and dietary restriction (Antosh et al., 2011; Galikova and Flatt, 2010; Wong et al., 2009). The *takeout* gene has also been shown to be associated with longevity, in connection to its role in feeding behavior (Antosh et al., 2011; Chamseddin et al., 2012; Galikova and Flatt, 2010). These studies have also made a connection between Juvenile hormone regulation of feeding behavior and longevity with *takeout's* role in these processes. The studies indicate collectively that Juvenile hormone and *takeout* might both act in these processes. Meunier and colleagues (Meunier et al., 2007) indicate a role for *takeout* in locomotor activity and the regulation of feeding behavior. The study indicates that to^1 mutant flies do not respond appropriately to food availability in food intake. The defect in food intake was shown to be based on the action of *takeout* on taste neurons; the sensitivity of to^1 gustatory neurons to sugars does not increase after starvation, as in wildtype flies.

Therefore, *takeout*, identified as a circadian regulated gene and initially shown to be involved in starvation response and feeding behavior in insects, was further shown to function in courtship behavior, locomotion, and longevity. How it might do so is unknown. Given its similarity to JHBPs this dissertation studies the intriguing possibility that Takeout may bind Juvenile hormone, and that the hormone may play a role in male courtship behavior.

The first JHBP was discovered in *Manduca sexta* (Kramer et al., 1974). Juvenile hormone is secreted into the hemolymph of insects. The authors hypothesized that in order to reach its

target cells, the hormone must be transported. Previous studies had shown the presence of proteins in the hemolymph of insects that are capable of binding to Juvenile hormone. A study by Kramer and colleagues (Kramer et al.,1974) experimentally demonstrated the presence of a JHBP of 30 KDa in the hemolymph that binds Juvenile hormone and its geometrical isomers with a high level of specificity (Kramer et al., 1974). The basic features of the hormone required to bind to JHBPs are the epoxide, the ester methyl function, and the aliphatic side chains (Goodman et al., 1976, Kramer et al., 1976, Gilbert et al. 1977). Several studies indicate the binding of every molecule of Juvenile hormone to a JHBP in the hemolymph (Touhara et al., 1993, Trowell, S.C.,1992; Hidayat, P., 1994).

JHBPs have been most extensively studied in Lepidoptera, specifically, in *Bombyx mori*, *Galleria mellonella*, and *Manduca sexta*. The JHBPs of Lepidopterans share 40-50% sequence identity, but no strong homology with other proteins. Takeout proteins only show up to 20% sequence similarity to JHBPs (Suzuki et al.,2011). Structural information of putative JHBPs are essential to determine the potential for a protein to actually bind Juvenile hormone. Crystal structures of JHBP of *Galleria mellonella* (Kolodziejczyk et al., 2008) and *Bombyx mori* (Fujimoto et.al.,2013) have been determined. The apo- and JH II- bound JHBP crystal structures as well as the JH III bound solution structures were determined. One such study suggests a mechanism of Juvenile hormone recognition by JHBP, ligand specificity, and a Juvenile hormone protection mechanism. The study reveals two hydrophobic pockets at each end of the elongated structure. One pocket binds Juvenile hormone while the other remains empty. The binding pocket is closed by hydrogen bonds at the center of the protein. These bonds were seen to be conserved in JHBPs while they were seen to be diversified in *takeout* proteins. Furthermore, the predicted Takeout structure apparently has a long elongated tunnel for hormone binding instead of the pocket

structure. Suzuki and colleagues (Suzuki et al.,2011) suggest that the uptake and release of Juvenile hormone is regulated by the opening and closing of an α-helix on the Juvenile hormone-binding pocket. This α-helix functions as a gate to sense ligand entry. JHBPs appear to recognize the structural features of the Juvenile hormone molecule with high specificity by hydrogen bond networks and hydrophobic contacts (Suzuki et al.,2011). Based on the above observations, the authors propose a mechanism for JHBP-mediated Juvenile hormone transport in the hemolymph from the site of synthesis (Corpora allata) to the target cells. According to this proposed mechanism, the protein equilibrates between a gate (α-helix) open form and a gate closed form when in the unbound state. At close proximity to the Corpora allata cell membrane, the gate of the JHBP opens due to decrease in dielectric constant and binding to Juvenile Hormone occurs in the hemolymph. The binding results in the formation of hydrogen bonds between the N and C terminal tails resulting in the fully gate-closed state with the hormone buried in the protein. The JHBP-JH-bound protein is transported in the hemolymph. When the bound protein reaches the target cells, the gate opens due to a decrease in dielectric constant to release the hormone to the target cell (Suzuki et al.,2011). They hypothesize that the hormone is taken up by the target cells by the use of a transporter (Suzuki et al.,2011). However, this mechanism is not yet demonstrated. Additionally, the mechanism by which the JHBP-bound Juvenile hormone recognizes the target cells is not known.

At present, the crystal structure of only one protein of the Takeout family has been determined, that of *Epiphyas postvittana* EpTo1 (Hamiaux et al., 2009). The protein shows a 45 Å long hydrophobic internal tunnel that extends the whole length of the protein and accommodates a bound ligand. The protein was a recombinant protein expressed in E.coli. The bound ligand was revealed to be *E.coli*-derived Ubiquinone-8. This study is the first evidence of Takeout being a

ligand carrier. However, the nature of the ligand-binding pocket is different from those of JHBPs investigated, indicating that Takeout proteins may carry a different type of ligand and not Juvenile hormone. However, since the protein was expressed in E. coli which lack eukaryotic protein modification enzymes, it is difficult to know how close this structure is to that of *Drosophila* Takeout in the hemolymph. Furthermore, the *Epiphyas postvittana* Takeout protein is phylogenetically far apart form *Drosophila melanogaster* Takeout. The study also indicates other differences between Takeout and JHBPs of Lepidoptera. EpTo1 contained only one N terminal disulfide bond as opposed to JHBPs that have two, as does *D. melanogaster* Takeout. The EpTo1 protein has the same α/β wrap fold observed in JHBPs suggesting a common fold for the JHBP/To superfamily. However, Takeout proteins share limited sequence conservation, implying that their functions depend on the ligands bound and their specificity (Du et al., 2003; Fujikawa et al., 2006; Hagai et al., 2007; Hojo et al., 2005; Saito et al., 2006). The long internal tunnel in EpTo1 suggests that it is less likely to be a JHBP (Hamiaux et al., 2009).

Taken together, it remains an open question whether Takeout is a JHBP. However, this knowledge is of critical importance to understanding the role of Takeout. For this reason, it was a goal of this thesis to determine whether Takeout binds Juvenile hormone.

1.3. Juvenile hormone

1.3.1. The Juvenile hormones

Juvenile hormones were initially identified by Wigglesworth in 1936. He discovered that the hormone has two major roles in the life history of an insect: to prevent metamorphosis and

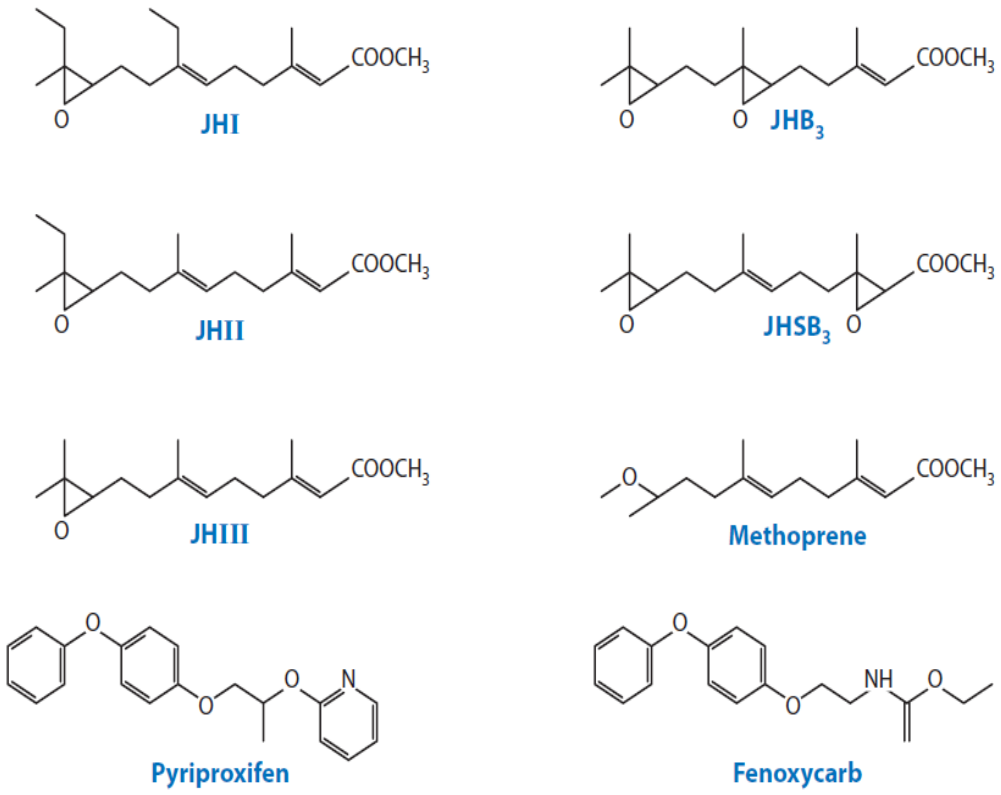
regulate reproduction. The hormone is called the 'status-quo' hormone based on its vital role of preventing precocious metamorphosis during larval life (Hartfelder, 2000).

The Juvenile hormones are unique sesquiterpenoid methyl esters that are principally known for their role in regulation of insect development. They function in embryonic development, repress metamorphosis, and induce vitellogenin synthesis and pheromone production (Nijhout, H.F., 1994). Juvenile hormone is also involved in generating polymorphism in aphids and social insects. The hormone has been considered an exclusive insect hormone.

Six forms of Juvenile hormone have been characterized. JH I and JH II have been identified in Lepidoptera. JH III has been found to be present in all insects. JH 0 and 4-methyl-JH I appear to be exclusive to Lepidoptera, while Diptera produce the 6.7- epoxide of JH III, JHB III (Belles et al., 2005), JH III and Methyl Farnesoate (Richard et al., 1989). JHB III appears to be the predominant sesqueterpenoid produced by the Corpora allata of higher Diptera (Richard et al., 1989).

The structures of Juvenile hormones and their mimics are shown in Figure 5 (Jindra et al.,2013).

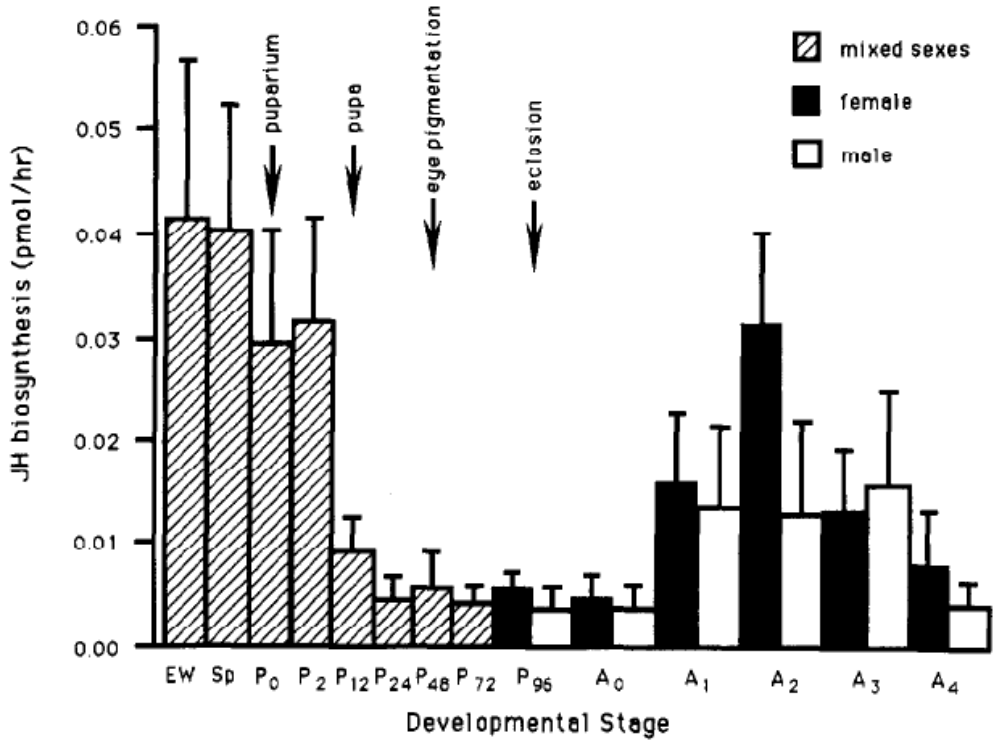
Methoprene and Pyriproxifen have been used previously to impose Juvenile hormone effects on insects (Abdou et al., 2011; Adam et al., 2003; Barry et al., 2008; Godlewski et al., 2006; Miura et al., 2005; Wilson and Fabian, 1986).



Jindra et al., 2013

Figure 5: Juvenile hormones and common analogs. The structures of major naturally occurring Juvenile hormones as well as the most commonly used Juvenile hormone mimics Methoprene, Fenoxycarb, and Pyriproxifen (Jindra et al. 2013).

Juvenile hormone biosynthesis was shown to be significantly higher in the larval stages of insects when compared to the pupal stages and adult stages (Dai and Gilbert, 1991) (Figure 6). However, it is present in adult males and females.



Dai and Gilbert, 1991

Figure 6: Juvenile hormone biosynthesis in different stages of the *Drosophila* life cycle. Juvenile hormone biosynthesis *in vitro* by the brain ventral ganglion ring gland complex or adult Corpora allata with their connections intact, during the larval-pupal- adult metamorphosis of *Drosophila melanogaster*. (Dai and Gilbert, 1991).

1.3.2. Juvenile hormone bio-synthesis

Juvenile hormone is synthesized in insects through the Mevalonate pathway (Figure 7). The pathway is based on the reductive polymerization of Acetyle Co-A which leads to isoprenoid compounds, including cholesterol. However, insects do not synthesize cholesterol *de novo* (Clark

and Block, 1959) as they lack the genes encoding the enzymes of the sterol branch (Santos and Lehmann, 2004). The Mevalonate pathway takes a separate branch at Farnesyl-PP (farnesyl di-phosphate) to proceed through Farnesoic acid to produce Juvenile hormone although the preceding steps are identical to cholesterol synthesis (Belles et al., 2005).

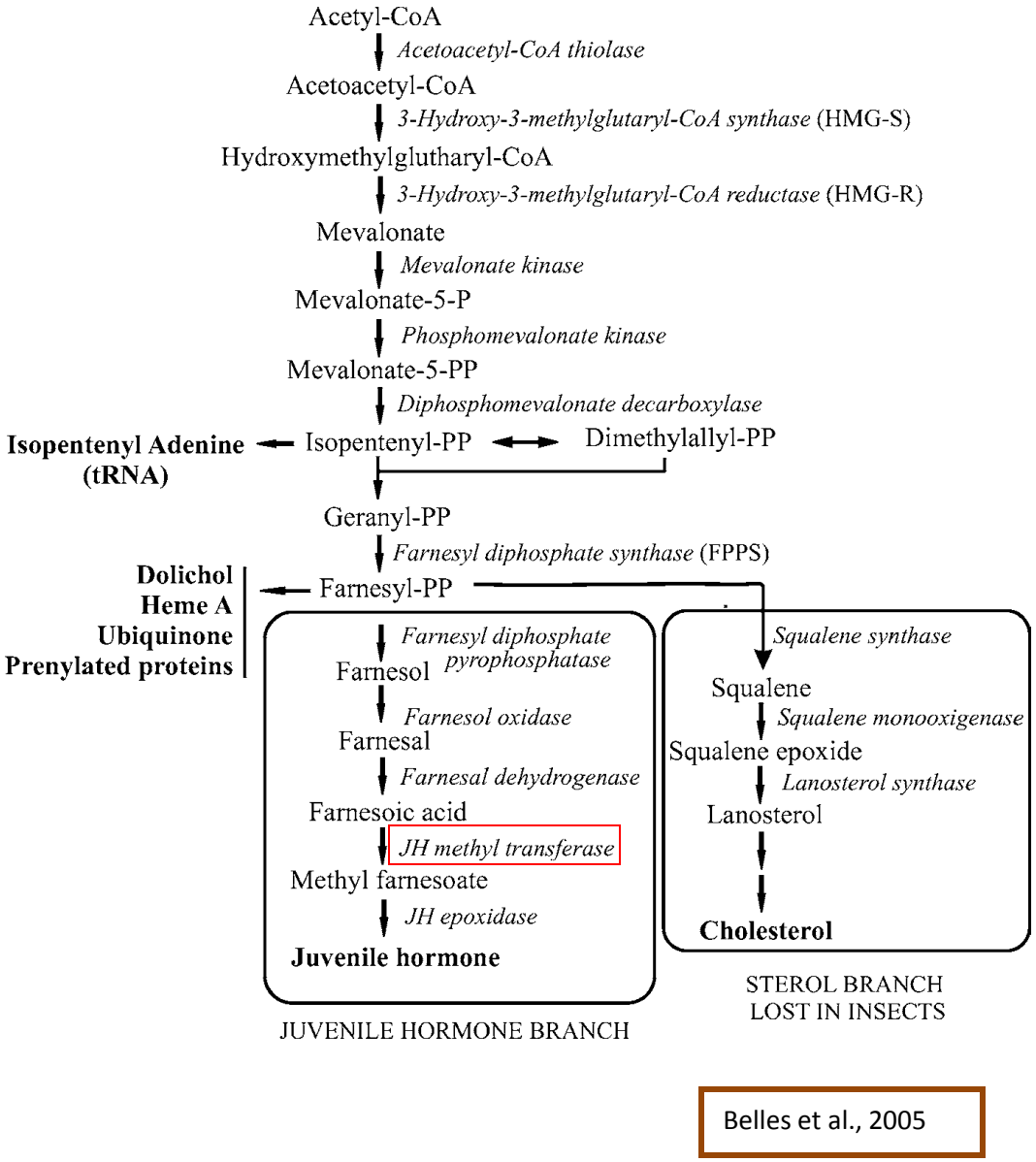


Figure 7: The Mevalonate pathway and Juvenile hormone or cholesterol biosynthesis. *Juvenile hormone Acid Methyl Transferase (JHAMT),* the enzyme that catalyzes the conversion of Farnesoic acid to Juvenile hormone is indicated (Belles et al., 2005).

1.3.3. Juvenile Hormone Acid Methyl Transferase (*JHAMT*)

JHAMT transfers a methyl group of S-adenosyl-L-Methionine (SAM) to Farnesoic acid (Belles et al., 2005). The enzyme is involved in the conversion of Farnesoic acid to Methyl Farnesoate, which is subsequently converted to Juvenile hormone by Juvenile hormone epoxidase (Belles et al., 2005). The first *JHAMT* cDNA was cloned from the Corpora allata of *Bombyx mori* (Shinoda and Itoyama, 2003). The cDNA codes for a 278 aa 32.5 KDa protein. The predicted amino acid sequence contains a conserved SAM-binding site. A recombinant *Bm*JHAMT protein that was expressed in *E.coli* was able to catalyze the conversion of Farnesoic and Juvenile hormone acids I, II and III to their methyl esters in the presence of SAM. This functional assay confirmed the identity of *Bm*JHAMT. Ortholog *JHAMT*s have been found for *Drosophila melanogaster* (*Dm*JHAMT) and *Anopheles gambiae* (*Ag*JHAMT). The *Bm*JHAMT gene was seen to be expressed in the Corpora allata from the first to the penultimate larval stage. The rapid decline in levels of this enzyme occurs at the final instar. The transcriptional suppression of *Bm*JHAMT gene expression is crucial to terminate Juvenile hormone biosynthesis in the Corpora allata (Shinoda and Itoyama, 2003).

The expression of *Bm*JHAMT was observed to be highly tissue-and-developmental-stage dependent. When the mRNA levels were absent in the final larval instar, trace amounts were detected in the testes, ovaries, and other tissue. The study indicates the possibility of *Bm*JHAMT activity in peripheral tissues and the synthesis of Juvenile hormone in these tissues in the pre-pupal period (Shinoda and Itoyama, 2003).

*Dm*JHAMT of *Drosophila melanogaster* was functionally characterized by expressing recombinant *Dm*JHAMT in *E.coli* and using it to catalyze Farnesoic acid to Juvenile hormone

acids in the presence of SAM (S-adenosyl-methionine) (Niwa et al., 2008). Specifically, it converts JHA III (Juvenile hormone Acid III) to Juvenile hormone and Farnesoic acid to Methyl Farnesoate. *Dm*JHAMT protein was shown to be predominantly expressed in the Corpora allata. Its expression profile correlates with Juvenile hormone titers (Niwa et al., 2008). The above authors used a *JHAMT-RNAi* transgene targeted to the Corpora allata to perform knockdown experiments. Contrary to expectations, these experiments did not affect the development of the flies. This suggests that the RNAi line used was very weak. However, the authors showed absence of α-JHAMT immunostaining in adult knockdown flies. It is possible that since Juvenile hormone levels in larvae are very high, the driver and RNAi used did not reduce the amount significantly enough to cause developmental disruption. In contrast, the authors observed that over-expression of the gene resulted in pharate adult lethal phenotypes similar to the application of Juvenile hormone analogues (Niwa et al., 2008). Several studies (Minakuchi et al., 2008a, Parthasarathy et al., 2009) performed RNAi against *JHAMT* in *Tribolium castanium*. Minakuchi and colleagues (Minakuchi et al., 2008a) have shown that RNAi against the functional homolog of *JHAMT* in *Triboilum castanium*, TcMT/ TcMethylTransferase in the larval stage resulted in precocious larval-pupal metamorphosis. This was also rescued by the application of the Juvenile hormone analog, Methoprene. Another study carried out by Parthasarathy and colleagues (Parthasarathy et al., 2009) performed RNAi of *JHAMT* in *Tribolium castanium* adults and observed a decrease in the size of the male accessory glands and the expression of accessory gland proteins. The study further indicates the reduction in Juvenile hormone titers in these beetles by the RNAi against *JHAMT*.

Therefore, the above studies clearly demonstrate the significance of the *JHAMT* gene with regard to Juvenile hormone synthesis. They demonstrate its expression in the Corpora allata as well as its targeting for the reduction in Juvenile hormone levels in insects.

The *JHAMT* gene (CG 17330) gene is located on the left arm of the 2nd chromosome.

1.3.4. The Corpora allata

The Corpora allata were first described in the literature by J. Müller in 1828 as organs in cockroaches. They were then thought to be pharyngeal bodies that innervated the oesophagus. In 1899, these organs were called the Corpora allata by R Heymons, but believed to be sympathetic ganglia. In 1910, G. Police suggested that the organ was an endocrine organ in Phasmids. Nabert, 1913 stated that Corpora allata were glandular secretory organs. This was confirmed by Ito, 1918, mentioning that they functioned in the adult moth as well.

Burtt, (in 1937 and 1938) described the organ in higher Diptera and discovered that these glands were a part of the Weissman's Ring, with is currently known as the Ring Gland (Burtt and Hadorn, 1937). It is currently known that the larval Ring Gland is composed of three structures, the Corpora allata, the Prothoracic gland and the Corpora cardia (Dai and Gilbert, 1991).

Wigglesworth (1935) showed that the Corpus allatum was the source of an inhibitory hormone that prevented metamorphosis in young larvae based on studies on *Rhodnius prolixis*.

The inhibitory regulation of the Corpora allata appears to occur by allatostatins, (a category of neuropeptides) and neurotransmitters. Stay and colleagues (Stay et.al., 1991) established a inhibitory control of the organ by allalostatin in the cockroach *Diploptera punctata*. These

allatostatins were thought to be delivered to the organ from the brain via axons or the hemolymph. In *Manduca sexta*, the negative regulation of the Corpora allata appears to occur via allatostatins (Zitnan et al., 1995) and allatinhibin (Bhaskaran et al., 1990). Since then, the functions of allalostatins have been established for a variety of insects. Cerebral allalostatins have been identified in *Drosophila* (Altaratz et al., 1991; Richard et al., 1990).

Stimulatory factors that regulate the Corpora allata (allalotrophins, ATHs) were isolated from Lepidoptera, the moth *Galleria mellonella* (Bogus and Scheller, 1994, 1996) and from Orthoptera (crickets and locusts) (Applebaum et al., 1990; Lehmberg et al., 1992; Lorenz and Hoffmann, 1995). The *Manduca sexta* allalotrophin has been structurally characterized (Kataoka et al., 1989).

In *Drosophila*, the ventromedial neurons are neurosecretory neurons (NSNs) innervating the ring gland (Horodyski et al., 1993). Siegmund and Korge (Siegmund and Korge, 2001) identified peptidergic brain neurons innervating the ring gland in *Drosophila*. Eleven groups of NSNs and their target tissues were identified. Five neurons of the lateral protocerebrum appear to directly innervate the Prothoracic gland or Corpora allata cells and are believed to directly regulate ecdysteroid and Juvenile hormone synthesis. The Corpora allata is innervated by only three of these; a single CA-LP 1 and two CA-LP 2 neurons.

The Corpora allata organ has been genetically ablated in several studies of *Drosophila* in order to disrupt the biosynthesis of Juvenile hormone (Gruntenko et al., 2010; Gruntenko et al., 2012) These studies have demonstrated the reduction of JHAMT activity and increase in Juvenile hormone hydrolyzing activity upon genetic ablation. The reduction of Juvenile hormone levels

by Corpora allata ablation was demonstrated by its effect causing abnormal development of the ovaries in females. (Gruntenko et al., 2010).

The above studies confirm the Corpora allata as the site of Juvenile hormone synthesis while establishing the regulatory mechanisms acting upon the organ. They also show that genetic ablation of the Corpora allata has been used as a mechanism of disrupting Juvenile hormone synthesis in *Drosophila*.

1.3.5. Functions of Juvenile hormone

1.3.5.1. Juvenile hormone in development

Juvenile hormone regulates metamorphosis and reproduction. Insects complete all growth before metamorphosis. The larval and pupal stages proceed from one to the next through a series of molts/ shedding of the exoskeleton. This growth and molting are caused and regulated by two major hormones; Juvenile hormone and Ecdysone. (Riddiford, 2012 Howard Berns lecture). Juvenile hormone is present in the larva and permits Ecdysone action to initiate molts but prevents Ecdysone from initiating genetic programs required for metamorphosis. Studies on *Manduca sexta* have shown that at the final larval instar, the Juvenile hormone titer declines to undetectable levels due to the cessation of production by the Corpora allata and due to the appearance of Juvenile hormone esterase. This consequently leads to the secretion of Prothoracicotrophic hormone (PTTH) from the brain and the secretion of a small amount of Ecdysone from the Prothoracic glands. In the absence of Juvenile hormone, Ecdysone causes cessation in feeding and onset of wandering to cause the pupal stage. The presence of Juvenile

hormone prevents pupal commitment by activating a downstream transcription factor *Krüpple homolog 1* (*Kr-h1*) that in- turn inhibits the 'pupal specifier' *Broad* (*br*). In the absence of Juvenile hormone, Ecdysone activates the expression of *br* resulting in pupal commitment. Juvenile hormone reappears during the pre-pupal period at the time of larval pupal molt together with the Ecdysone rise, to prevent precocious metamorphosis into the adult, thereby maintaining the pupal stage. Juvenile hormone disappears at the time of pupal ecdysis to permit the Ecdystroid rise to cause metamorphosis and adult commitment (Figure 8) (Riddiford, Howard berns lecture. 2012).

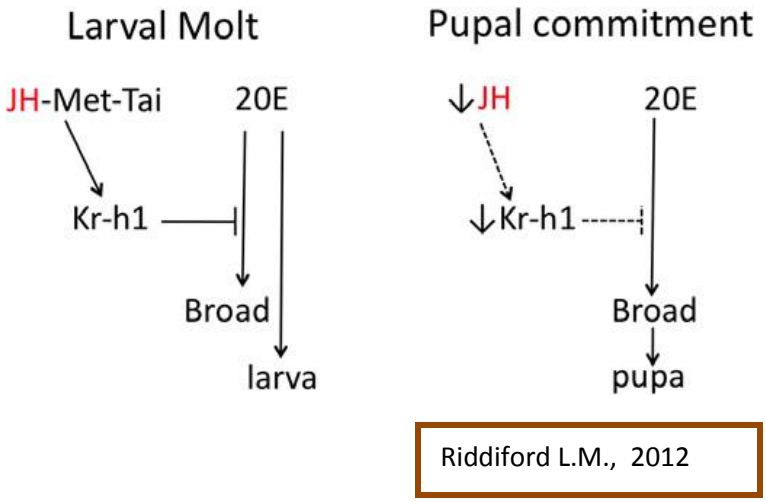


Figure 8: Juvenile hormone and Ecdysone in the control of development. Model for the molecular basis for the control of molting and metamorphosis by Juvenile hormone and Ecdysone (Riddiford , Howard berns lecture. 2012).

1.3.5.2. Juvenile hormone in the adult insect

Juvenile hormone is well known to orchestrate various processes in the adult insect (Bowens M., 2004; Wyatt and Davey, 1996; Raikhel et.al, 2005). It is involved in vitellogenesis, oogenesis, and post-mating behavior in females, as well as accessory gland protein (sex peptide) production in males. In addition to reproductive functions, it has recently been suggested that Juvenile hormone is involved in longevity and stress resistance in insects. These additional functions are described below.

The gonadotrophic role of Juvenile hormone was established by Wigglesworth in *Rhodnius prolixus.* Insect Vitellogenins are members of a large family of lipid-binding proteins. The titers of these proteins secreted from the fat body of the adult female account for 50% of their total hemolymph proteins (Hartfelder, 2000). Juvenile hormone is involved in vitellogenesis in the feeding adult female. Vitellogenesis is the synthesis of Vitellogenin (Vg)/ yolk protein in the fat body of the female and its uptake by the growing oocyte. Recent studies in *Tribolium* have shown that Vg synthesis in the fat body requires both feeding and Juvenile hormone (Sheng et al., 2011). Vg expression is repressed by the FOXO transcription factor. Its loss from the nucleus is mediated by the Insulin signaling pathway. Juvenile hormone stimulates the increase in ILP2 (Insulin Like Peptide 2) mRNA in the fat body and brain, while feeding stimulated the increase of ILP3 mRNA in the fat body. The upregulation of ILP2 was shown to occur by Juvenile hormone acting through the Met transcription factor (Sheng et al., 2011).

In *Drosophila* Juvenile hormone affects vitellogenesis, involving the synthesis and secretion of yolk proteins (YP1, YP2 and YP3) by the fat body and ovarian follicle cells and the uptake of yolk protein into the oocytes (Bownes, M., 1982, 1989; Bownes et al. 1996; Handler, A.M., 1977;

Jowett and Postlethwait, 1980; Wilson, T.G. 1983). Juvenile hormone primarily regulates Vg uptake into oocytes (Soller et al., 1999), but the mechanisms are unknown. Wilson and Ashok (1998) have shown a delay in egg maturation and reduced fecundity in *Met* null mutants. Mutations in *apterous (ap*) cause sterility due to non-vitellogenic ovaries (Altaratz et al., 1991; Dai and Gilbert, 1993; Postlethwait and Handler, 1978; Postlethwait and Weiser, 1973). *Met* has been shown to bind Juvenile hormone, and *ap* mutations affect Juvenile hormone levels.

In sexually mature females of *Drosophila*, egg production (oogenesis) occurs continuously. The sexually mature virgin females rarely deposit eggs, as the egg development is terminated at stage 9 of oogenesis in a process involving apoptosis and egg resorption requiring high levels of 20E (20-hydroxyecdysone). Sollar and colleagues (Soller et al., 1999) propose that both Juvenile hormone and 20E act together with the accessory gland protein Sex-peptide after its transfer from the male to the female at mating. Sex-peptide stimulates Juvenile hormone synthesis in the female, and consequent vitellogenesis and oocyte progression through the stage 9 checkpoint, while reducing 20E levels. Previous research by Manning (Manning, 1966) has proposed that Juvenile hormone is involved in the maturation of female receptivity. Wicker-Thomas and Chertemps (2010) suggest that the role of Juvenile hormone in female receptivity may be through its involvement in the synthesis of female pheromones.

The production of male accessory gland proteins is known to be under the control of Juvenile hormone. Studies by Yamamoto and colleagues (Yamamoto et al., 1988) showed that either mating or low doses of Juvenile hormone stimulate Acp protein production in the accessory glands. These proteins are produced and deposited in the female reproductive tract during mating. They function to reduce female receptivity, stimulate oogenesis and oviposition, provide

antimicrobial and nutritive properties, and promote sperm storage in the female (Wilson et al., 2003). Sex-peptide, an accessory gland protein, stimulates Juvenile hormone synthesis in females after mating, thereby promoting oogenesis and post-mating behavior (Moshitzky et al., 1996). Wolfner and colleagues (Wolfner et al., 1997) have performed studies which indicate that Juvenile hormone and 20E are required in males to stimulate the synthesis of Acps to replenish the proteins lost after transfer to the female at mating.

In addition to the above more well known functions of Juvenile hormone in the adult insect, a few studies have indicated some additional functions as described below.

In butterflies (Lepidoptera), several species of grasshoppers and *Drosophila* features of reproductive diapause (arrested oogenesis, stress resistance, and negligible senescence) appear to be partly controlled by Juvenile hormone and Ecdysone (Richard et.al., 2001; Tartar and Yin, 2001; Tartar et al. 2001; Saunders et al., 1990; Tu et al. 2005). The above studies indicate that diapausing *Drosophila* females have reduced Juvenile hormone levels and have a ovarian arrest. This can also be rescued by Methoprene, confirming the function of Juvenile hormone.

Interestingly, Juvenile hormone has been shown to be a pro-aging hormone as it decreases life span (Tartar and Yin, 2001). The above study indicates an increase in life span by the surgical removal of the Corpora allata in grasshoppers and butterflies. The pro-aging function of Juvenile hormone has also been shown in *Drosophila* by the Methoprene treatment of long-lived flies shortening life expectancy towards those of wildtype (Tartar et al. 2001). More recently, Galikova and Flatt (Galikova and Flatt, 2010) have suggested that Juvenile hormone and Ecdysone regulate lifespan by regulating the expression of *takeout*. Additionally, Juvenile hormone might function in stress resistance. Flatt and colleagues (Flatt et al., 2005) mention

that reproductively dormant *Drosophila*, where Juvenile hormone levels are low, show an increased resistance to oxidative and heat stress.

These studies project Juvenile hormone as a very active and important hormone in the adult insect, with many functions concerning reproduction, longevity and stress resistance.

1.3.6. Downstream genes of the Juvenile hormone signaling pathway in insect development

1.3.6.1. *Methoprene tolerant* (*Met*)

Met is considered the most likely Juvenile hormone receptor candidate. It has been identified in a genetic screen for mutants resistant to the Juvenile hormone agonist, Methoprene (Wilson and Fabian, 1986). Methoprene has previously been identified as a Juvenile hormone analog by various studies (Cerf and Georghiou, 1972; Staal, 1975; Sehnal and Zdarrek, 1976). The mutation was called Methoprene tolerant *(Met)* (Wilson and Fabian, 1986). *Met* is a bHLH transcription factor (Ashok et al., 1998) and has been shown to bind Juvenile hormone (Shemshedini and Wilson, 1980). The *Met* protein was observed to be located in the nucleus by immunochemical studies done on *Drosophila* embryos, larvae, and early pupae, as well as in ovaries and male accessory glands in adults. The expression was observed in all stages of development (Pursley et al., 2000). Recombinant Met protein prepared in an *in vitro* transcription-translation system binds Juvenile hormone with high affinity consistent with the physiological Juvenile hormone concentration (Miura et al., 2005). In transient transfection assays, using *Drosophila* S2 cells, the study demonstrated that the yeast GAL4-DNA binding domain fused to *Met* exerted Juvenile

hormone or Juvenile hormone acid-dependent activation of a luciferase reporter gene, verifying the binding between Juvenile hormone and *Met*.

The *Met* null allele was found to be fully viable (Wilson and Ashok, 1998) *Met* forms homodimers or forms heterodimers with Germ Cell Expressed (*Gce*). Juvenile hormone reduces their dimerization. In the presence of Juvenile hormone or a Juvenile hormone agonist, the binding between *Met-Met* and *Met-Gce* were seen to be drastically reduced (Godlewski et al., 2006). *Met* overexpression leads to precocious and enhanced programmed cell death (PCD) in larval tissue (Liu et al., 2009) leading to increased mortality during larval development (Barry et al., 2008). During larval-pupal transition, *Met* and *Gce* mediate Juvenile hormone action to prevent 20E triggered caspase-dependent PCD in the larval fat bodies (Liu et al., 2009) and the optic lobe of the adult brain (Riddiford et al., 2010). Mutations lacking either one of *Met* or *Gce* showed normal progression through metamorphosis. However, when both proteins were absent, the mutants pupariate, but die at head eversion. They do not reach adult stage. The study suggests that *Met* and *Gce* redundantly transduce Juvenile hormone action to induce *Kr-h1* expression and consequently inhibit *br* expression (Abdou et al., 2011). Met was shown to interact with Ecdysone Receptor (EcR) and Ultraspiracle (USP) in a ligand-independent manner, using two hybrid pull down assays (Bitra and Palli, 2009).

The overexpression of *Met* resulted in increased sensitivity to Methoprene, i.e. higher susceptibility to morphogenetic and toxic effects of Methoprene, consistent with the hormone-binding property of *Met* (Barry et al., 2008). In Met^{27}, a null allele of *Methoprene-tolerant*, in addition to Juvenile hormone resistance, oogenesis of mutant females is reduced (Wilson and

Ashok, 1998), and males show reduced protein accumulation in the accessory glands. The males were seen to court and mate (Wilson et al., 2003).

Juvenile hormone (JH III), as well as its biologically active mimics, Methoprene and Pyriproxifen, were shown to specifically bind Met through its C-terminal PAS domain. It has been shown that Juvenile hormone signals through the nuclear receptor protein FTZ-F1 to interact with Met and Gce. Subsequent transcription occurs through the FTZ-F1 response element to activate a nuclear receptor protein E75A (Dubrovsky et al., 2011). When individual amino acids that were predicted to form the ligand-binding pocket were substituted with others, the binding between Juvenile hormone or its mimics with *Met* was reduced. Furthermore, these mutations did not reduce *Met-Met* binding, but reduced the ligand dependent dissociation of the *Met-Met* complex and the ligand-dependent interaction of *Met* with *Taiman* (Charles et al., 2011). *Taiman* was shown to be the *Drosophila* homolog of a bHLH transcription factor, FICS in Aedes egypti (Li et al., 2011), and SRC in *Tribolium castanium* (Zhang et al., 2011). *Met* and Broad (*br*) interact with each other to regulate expression of downstream genes (Wilson et al., 2006). Met mutants were also shown to have reduced protein accumulation in their accessory glands and were shown to court and mate wildtype females with reduced vigor when compared to wildtype males. This observation hypothesizes a role for Juvenile hormone in courtship behavior (Wilson et al., 2003).

In *Tribolium castanium*, *Met* functions in Juvenile hormone action in maintaining proper larval molting and preventing premature development of adult structures during larval-pupal metamorphosis (Konopova and Jindra, 2007; Parthasarathy et al., 2008). *Tribolium* contains only one *Met* gene. The suppression of its expression by double stranded RNA in the third or fourth

larval instars resulted in precocious metamorphosis. Also, when *Met* was knocked down by RNAi, the animals were observed to be un-responsive to Methoprene and Juvenile hormone (JH III) and the larvae appeared unable to ecdyse and arrested as premature pre-pupae. Therefore, the study demonstrates that *Triboilum Met* mediates Juvenile hormone response and is required for proper timing of entry to the metamorphic pupal program. Therefore, this study also supports the hypothesis that *Met* might serve as a Juvenile hormone receptor (Konopova and Jindra, 2007). Charles and colleagues (Charles et al.2011) showed that *Tribolium Met* binds Juvenile hormone and its mimics with high affinity through a conserved binding pocket within its PAS-B domain.

1.3.6.2. *Germ Cell Expressed* (*Gce*)

The first protein partner described for *Drosophila* Met was Gce (Godlewski et al., 2006). The finding in the above study, that upon contact with Met or Juvenile hormone JH III, both Met-Met complexes and Met-Gce complexes can dissociate, indicates a ligand dependent mechanism of action. Gce is highly similar to Met and is supposed to have arisen from gene duplication of Met (Baumann et al., 2010; Baumann et al., 2010).

1.3.6.3. *Ultraspiracle* (*USP*)

USP is a member of the nuclear receptor superfamily in invertebrates. It has high similarity to vertebrate Retinoid acid receptors (RXRs) and is found only in Lepdopterans and Dipterans of insects (Riddiford et al., 2001; Iwema et al., 2007). USP/RXR is best known as a heterodimeric partner of the Ecdysone Receptor (EcR), where Ecdysone binds the ligand 20E. When 20E is absent, USP/EcR heterodimeric receptor binds the EcRE (Ecdysone Receptor Element) in the promoters of Ecdysone-regulated genes and inhibits their transcription (Schubiger and Truman,

2000; Riddiford et al., 2001). The binding of Ecdysone to the ligand-binding domain of USP de-represses these genes and activates them.

Juvenile hormones are considered to have functional similarity to Retinoic acids. Similarly to Retinoic acid, Juvenile hormones are synthesized from the common isoprenoid precursor, Farnesyl diphosphate, through the Mavalonate pathway (Harmon et al., 1995). Additionally, Juvenile hormone is a sequeiterpenoid, which is chemically related to the vertebrate Terpene group, represented by Retinoic acid (Jones and Sharp, 1997). The above study shows that two active Juvenile hormone structures found in *Drosophila* (JH III and JH III Acid) bind the *Drosophila* USP and induce conformational changes and homo-oligomerization (Jones et al., 2001). Based on these data, Jones and colleagues proposed that USP is a receptor for Juvenile hormone. However, *in vivo* ligand trap studies in *Drosophila* indicate that only fenoxycarb (Juvenile hormone mimic) activates USP. The other Juvenile hormone mimics such as Methoprene did not activate USP (Palanker et al., 2006). Additionally, Jones and colleagues (Jones et al., 2006) found that Methyl Farnesoate, the immediate precursor of Juvenile hormone, binds USP at significantly higher affinity than JH III. It is hypothesized that Juvenile hormone alone acts through the USP homodimer, whereas the synergistic action of Juvenile hormone is through the USP/EcR heterodimer with Juvenile hormone binding to the same site in both dimers (Riddiford, 2008).

1.3.6.4. *Kruppel-homolog* (*Kr-h1*)

Kruppel homolog (*Kr-h1*) is a zinc-finger transcription factor. It has been documented in *Drosophila* that its expression is induced by Juvenile hormone (Minakuchi et al., 2008b). The authors identified *Kr-h1* in a genome wide analysis in *Drosophila* for Juvenile hormone-regulated

genes in the abdominal integument to which the Juvenile hormone mimic, Pyriproxifen was applied. The mis-expression of *Kr-h1* in the epidermal cells resulted in short or missing bristles. This was similar to the application of Juvenile hormone and the mis-expression of *br* in early adult development. This action of *Kr-h1* gave a prolonged expression of *br*, indicating that *Kr-h1* acts downstream of Juvenile hormone and upstream of *br*. Several *Kr-h1* mutations were shown to induce death at pre-pupae, which is accompanied by Ecdysone-dependent gene expression at onset of metamorphosis (Beck et al., 2004; Pecasse et al., 2000). In *Drosophila*, *Kr-h1* mRNA is present throughout life, but reduced at the final larval instar. It reappears at the pupal molt transiently as it is required for normal pre-pupal development (Pecasse et al., 2000). The loss of *Kr-h1* was seen to lead to the early appearance of *br* in the fat body at the molt into the third instar (Huang et al., 2011).

In *Tribolium castanium*, *Kr-h1* was shown to be activated by Juvenile hormone through Met. Here, it was shown to be a early response gene of Juvenile hormone that connects *Met* and *br* (Minakuchi et al., 2009).

1.3.6.5. *Broad* (*br*)

The gene *Broad* (previously known as the Broad complex in *Drosophila*), is an ecdysone regulated BTB and zinc-finger transcription factor. In flies, there are four isoforms and the loss of all four results in death at the time of pupariation (Jindra et al., 2013). The *br* gene is regarded the pupal specifier as mutants that lack all alleles remain at the final instar larval state (Emery et al., 1994; Kiss et al., 1988; Kiss et al., 1976). The gene is suppressed by Juvenile hormone through *Kr-h1* during the larval stages. The expression of *broad* occurs due to an early low level

burst of 20E at the start of pupariation. The expression of *broad* is switched off early in pupal development to permit the pupal –adult molt (Riddiford, L.M. Howard berns lecture. 2012).

1.3.6.6. *Apterous* (*ap*)

The gene *Apterous (ap)* encodes a developmental regulatory gene that has been shown in *Drosophila* to be a requirement in the larval and adult CNS for normal hormonal regulation of vitellogenesis (Cohen et al., 1992). The gene is required for normal wing development and survival (Wilson, T., 1980; Stevens and Bryant, 1985; Wilson, 1981). Many *ap* mutations decrease the rate of Juvenile hormone production in the Corpora allata (Altaratz et al., 1991).

The *ap* mutation leads to several phenotypes in the adult (Butterworth and King, 1965), among them non-vitellogenic oocyte development and failure of larval fat body histolysis. These appear to be due to a Juvenile hormone deficiency since they can be rescued by the application of the synthetic hormone (Gavin and Williamson, 1976; Postlethwait and Jones, 1978; Postlethwait and Weiser, 1973). A positive connection was observed between Juvenile hormone production and female receptivity (Ringo et al., 1991). Studies done by Altaratz and colleagues (Altaratz et al., 1991) indicate that the end product of adult female Corpora allata is JHB3 (Juvenile Hormone Bisepoxide 3) and that the ap^4 and ap^{56} mutations interfere with the terminal oxidase activity of Juvenile hormone synthesis, resulting in a reduced conversion of Methyl Farnesoate and JH III to JHB3. The study further shows that under normal wildtype conditions, the synthesis of JHB3 influences the brain to secrete neuroendocrine factors to regulate Corpora allata activity, acting as a negative feedback mechanism. *ap* mutant (ap^4) females were also shown to have reduced Juvenile hormone synthesis by other studies (Bodenstein, 1947; Handler and Postlethwait,

1977). These observations suggest that the *ap* gene product is involved in Juvenile hormone synthesis or secretion.

Several studies have been carried out on the *ap* mutants and male mating behavior (Ringo et al., 1992; Tompkins, 1990). The *ap* locus is essential for effective male courtship and timely loss of immature male sex appeal (Ringo et al., 1991). It has a profound effect on male mating success. Several reasons were suggested. *ap* is essential for wing development and thereby to effectively perform the courtship song. A previous study by Tompkin and colleagues (Tompkins, 1990) indicates a general sluggishness along with lack of co-ordination and paralysis that may contribute to reduced courtship.

1.4. Hypothesis and statement of the question

The similarity between JHBPs in insects and Takeout raises the possibility that Takeout may be a JHBP in flies. A JHBP has not yet been identified in *Drosophila*. Furthermore, based on an extensive body of previous research on courtship behavior in *Drosophila melanogaster* and Juvenile hormone and its functions in insects, along with the role for Takeout, a male preferentially expressed protein in adults, in courtship behavior, there exists a strong possibility that Juvenile hormone can play a role in male courtship behavior in flies.

Therefore, this study aimed to address the question of whether Juvenile hormone plays a role in male courtship behavior and explores if its mechanism is through the binding to Takeout.

The study consists of two aims addressing the following questions in *Drosophila melanogaster*-

- **Does Juvenile hormone play a role in courtship behavior?**

  This question was addressed by experiments aimed at reducing Juvenile hormone levels. This was done by universal and specific, as well as conditional expression of a RNA interference construct targeting *JHAMT*, and by genetic ablation of the Corpora allata, followed by the examination of the courtship behavior of these flies.

- **Does Juvenile hormone bind takeout?**

  This question was addressed by expressing Takeout *in vitro* using the Baculovirus expression system in insects and testing its binding of radiolabeled Juvenile hormone.

1.5. Gene expression systems used in this study

1.5.1. GAL4-UAS system for spatial control of gene expression

The GAL4-UAS system is the most widely used system in *Drosophila* to achieve spatially restricted gene expression (Brand and Perrimon, 1993). The authors developed a two-part system to target gene expression that is restricted to specific cells and tissues (Figure 9). The system expresses the yeast transcriptional activator *GAL4* in different tissues according to the requirements of the study. To activate the gene in a specific location, target genes-of-interest (UAS-GOI) were synthesized containing binding sites for the GAL4 transcriptional activator. The target gene (UAS-GOI) and the activator (tissue-specific promoter-*GAL4*) are placed in two separate transgenic lines and are then brought together by crossing the two lines. The required expression of the target gene is achieved in the progeny. This system can be used to express any

gene ectopically and can be applied to embyos, larvae, and adults (Brand and Perrimon, 1993). The system has several advantages. It allows the rapid generation of individual strains in which the GOI can be specified to different tissues or cell types; it allows separation of the target GOI from the transcriptional activator in separate parental lines that are therefore individually fully viable. Several drawbacks exist to this system. One major limitation is the inability to temporally induce the system (The TARGET system was later devised in flies to address this limitation). Many *GAL4* lines are dynamic in their expression pattern throughout development, making it difficult to determine if rescue experiments using *GAL4* represent a developmental or adult specific rescue. Also, many *GAL4* lines express early in development and therefore cannot be used to express toxic gene products (McGuire et al., 2004b).

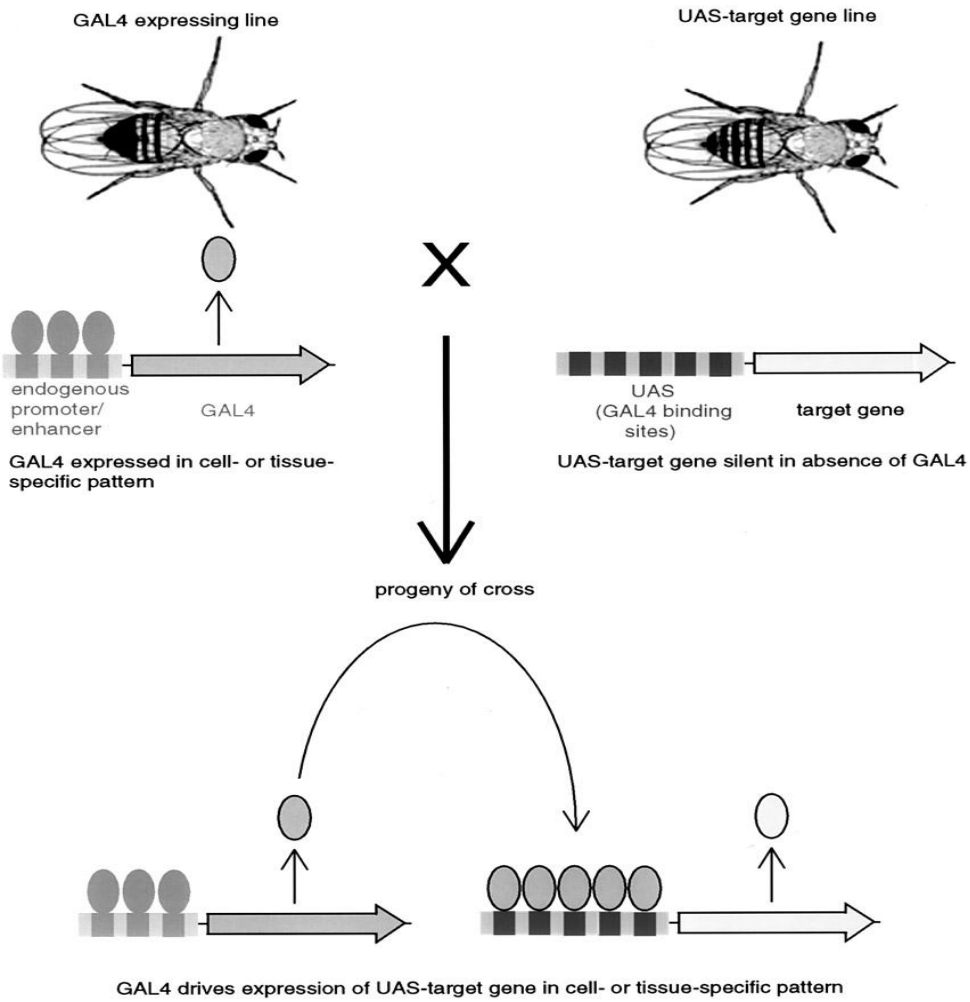


Figure 9: The principles of the GAL4-UAS system. The tissue specific *GAL4* activator and the target gene cloned to a *GAL4* responsive UAS sequence, are placed in separate transgenic lines and are brought together in their progeny, thereby activating the target gene in the location of interest (Phelps and Brand, 1998).

1.5.2. TARGET system for temporal and spatial control of gene expression

The TARGET (Temporal and Regional Gene Expression Targeting) system inserts the temporal gene expression capability into the GAL4-UAS system (Figure 10). The system was also introduced to *Drosophila* from yeast. It enables the induction of tissue-specific gene expression in a determined time or time span in the life cycle of the fly. The expression of the target gene (*UAS-GOI*) by the GAL4 activator is prevented until the determined time period of the fly's life cycle by the use of a temperature sensitive Gal80^{ts} protein. The *Gal80^{ts}* is driven universally in the body by the use of a Tubulin 1α-promoter (*tubulin promoter-GAL80^{ts}* transgene). The Gal80^{ts} protein performs optimal repression of *GAL4* activity at 19°C and de-repression begins at 30°C. Therefore, lower temperatures inhibit and higher temperatures permit transcription of *GAL4* and consequently the target gene of interest (UAS-GOI). TARGET additionally offers dose dependent repression of GAL4 activation of a gene of interest by the presence of multiple copies of GAL80^{ts} (McGuire et al., 2003; Roman, 2004). The TARGET system is advantageous in that it is induced by temperature and is therefore, rapid and easily controlled. However, elevated temperatures may not always benefit the flies and may have undesirable effects (Roman, 2004).

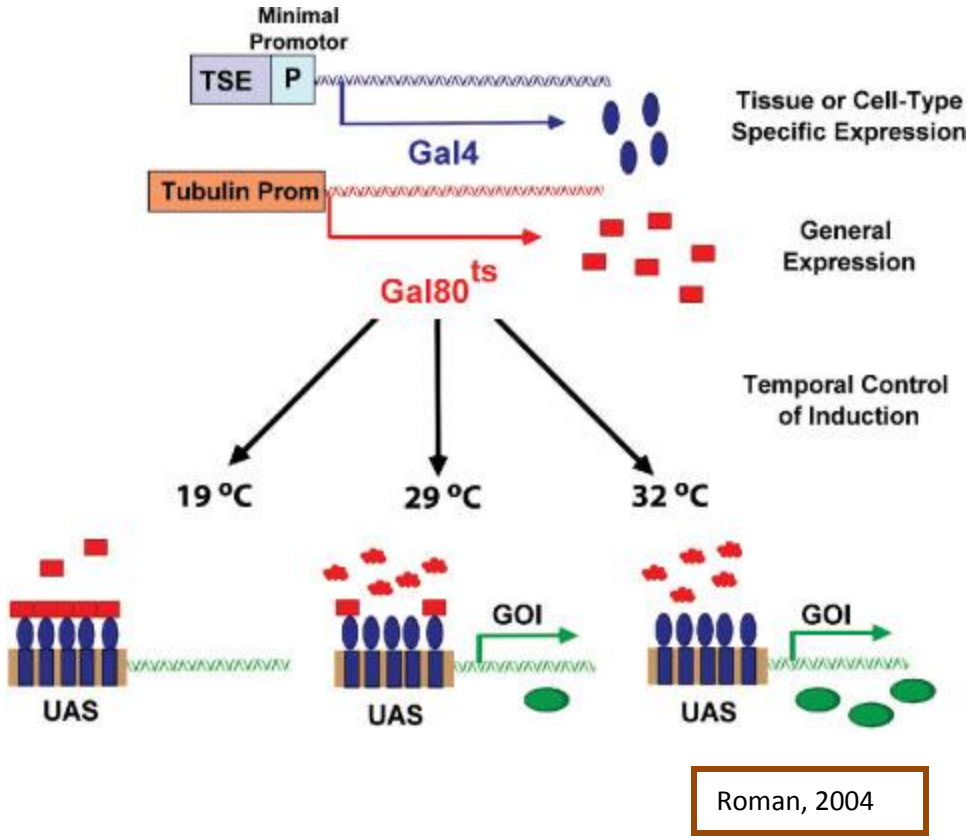


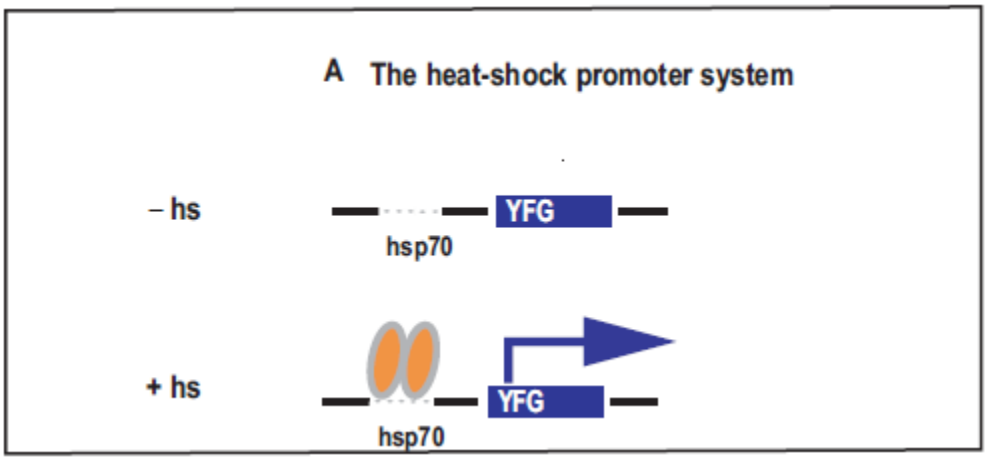
Figure 10: The principles of the TARGET system. Expression of *GAL4* and consequently the GOI is suppressed by an active universally expressed *Gal80^{ts}* protein at 19°C. The expression is permitted at 30°C or higher (Roman, 2004).

1.5.3. Heat shock system for temporal control of gene expression

Heat shock proteins (e.g. *hsp70*) are induced in *Drosophila* cells when the flies are exposed to a high temperature of 29-37°C. A maximal level is achieved at 37°C. Heat shock proteins are defined as those whose expression is significantly increased at high temperature. The most highly conserved of these proteins is *hsp70* (Lindquist, 1986). The *hsp70* heat shock promoter, when cloned upstream of a GOI, can be induced by heat shock to control the temporal pattern

of expression of the GOI (Figure 11). This enables the controlling of the timing of expression of the gene, albeit ubiquitously. The method also enables the regulation of the level and persistence of the transgene expression by changing the intensity and length of the heat shock (McGuire et al., 2004b).

Therefore, the cloning of a heat-inducible *hsp70* promoter upstream of a *GAL4* sequence enables the temporal induction of *GAL4* and consequent temporal and special expression of the *GOI*, albeit ubiquitously. Several drawbacks also exist to this approach, i.e. that a basal expression of heat shock proteins occur under non-induced conditions and that the heat shock used to induce the system can cause detrimental effects on the flies. The system is also ubiquitously expressed and not tissue specific.



McGuire et al., 2004

Figure 11: The principles of heat-shock system. The *hsp70* promoter is induced by exposure of the fly to heat shock (McGuire et al., 2004a).

1.5.4. Targeted cell ablation

Targeted cell ablation can be used to confirm the requirement of a cell for a biological process. By targeting a particular cell for ablation using the GAL4-UAS system, a non-invasive cell ablation can be achieved. Targeted cell death has been achieved using a variety of target genes (*UAS-GOI*); *UAS-reaper*, *UAS-HID*, *UAS-DTI*, *UAS-ricin A* and *UAS-Grim* are some of them (Phelps and Brand, 1998).

Two cell ablation approaches were utilized in this study; the HID and DTI systems.

a) HID system. Used to perform targeted apoptotic cell death. *UAS HID* has been used in previous studies by co-expression of *UAS-HID* and another cell death construct, *UAS-reaper* to achieve cell death of the corpora allata in flies (Gruntenko et al., 2010; Gruntenko et al., 2012).
b) Diphteria Toxin. Although not a specific cell death gene, expression of DTI in cells results in toxicity by the attenuation of protein synthesis. Diptheria Toxin A (DTA) is an inhibitor of protein synthesis by ribosylating EF-2 (Wilson and Collier, 1992). It has been used in several previous studies (Chow et al., 2011; Ghosh et al., 2011; Han et al., 2000; Kunes and Steller, 1991; Wilson and Collier, 1992). As the toxicity of DTA is extreme, (Yamaizumi et al., 1978) an attenuated mutant of DTA, DTI is used (Bellen et al., 1992).

1.5.5. Baculovirus Expression Vector System (BEVS)

The Baculovirus Expression Vector System (BEVS) is a powerful, widely used system for the routine expression of foreign genes in a eukaryotic *in vitro* system. A helper-independent

system, it is able to express genes from a variety of sources, including fungi, bacteria, insects, and mammals. The BEVS includes a variety of transfer vectors and simple methods for isolation and quantification of viruses (Kost et al., 2005). Several factors have contributed to the popularity of the BEVS. Firstly, it is a eukaryotic system, as opposed to a bacterial system. Therefore, it uses many of the post-translational modifications, protein processing, and transporting present in the higher eukaryotic systems. Secondly, it is helper-independent, facilitating the propagation of the virus to high titers in suspension cultures. Therefore, large amounts of proteins can be obtained with relative ease. A majority of the protein remains soluble in insect cells, as opposed to the insoluble proteins produced by bacterial systems (Murphy et.al., 2004). This would facilitate relatively straightforward secretion of secretary proteins out of the cell. As the viral genome is large, it can accommodate large regions of foreign DNA. The BEVS typically produces over-expressed proteins with proper folding, bond formation and oligomerization (Kidd and Emery, 1993). The system is capable of performing several post-translational modifications, resulting in the expressed protein being functionally and structurally similar to the native protein. The high expression levels characteristic of the BEVS system, as well as the capacity to carry large DNA inserts make it an attractive expression system (Baixeras et al., 1990; Brandt-Carlson and Butel, 1991; Caroni et al., 1991; Christensen et al., 1993; Hsu et al., 1991; Mattion et al., 1991). Finally, the viruses are non-infectious to vertebrates (except those that have been especially engineered to infect mammalian cells). Their promoters are inactive in mammalian cells.

Baculoviruses (family *Baculoviridae*) are large double stranded viruses. These highly species-specific viruses use a variety of insects as their natural hosts (Mathews, R.E.F., 1982). Currently, the most extensively used Baculovirus strain is AcMNPV, (Murphy et.al., 2004). Infection is by

endocytosis after which the virus enters the nucleus and replicates (Figure 12 A), producing occluded or non-occluded viruses. The occluded virus particles are embedded in proteinaceous viral occlusions, made of the polyhedron protein (29KD). The polyhedron protein functions to protect the virus from decomposing host cell enzymes when the host cell is killed by infection of the virus. The protein is essential for survival of the virus only in nature and not in tissue cultured cells (Murphy et.al., 2004).

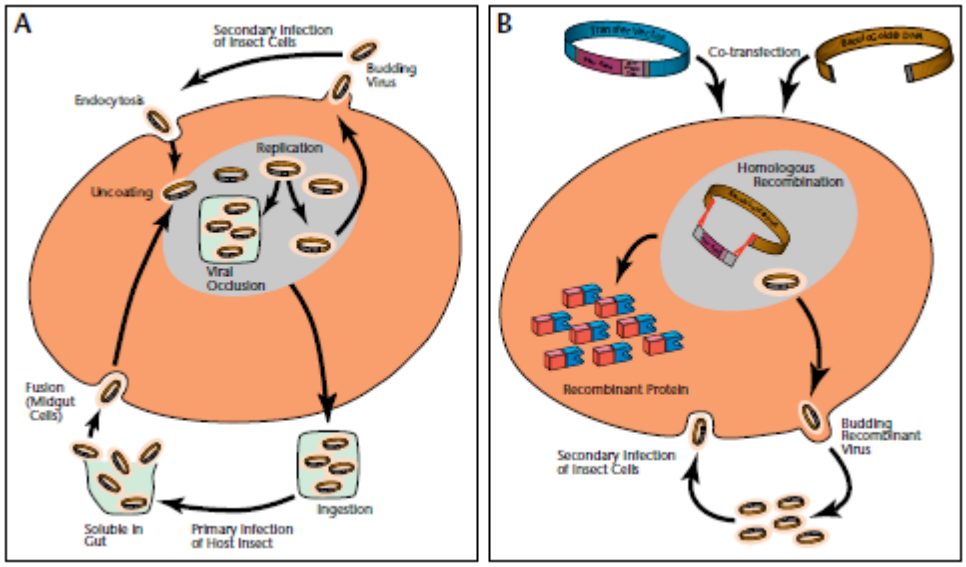
The BEVS uses the polyhedron promoter of the virus for several purposes: the promoter has very high activity facilitating high gene expression levels at the late stages of infection; the polyhedron gene is not essential for the infection and replication of the virus and therefore, helper viruses are not required; plaque of viruses without the polyhedrin coating are distinguishable from those who have the coating. Recombinant viruses are produced by replacing the polyhedrin coding region with the GOI. Recombinant viruses can be identified by the absence of the coating (Murphy et.al., 2004).

Many Baculovirus transfer vectors have been constructed using AcNPV DNA. These vectors all contain an *E.Coli* origin of replication, an Ampicillin resistance marker, a polyhedron promoter, a cloning region downstream from the promoter to insert foreign genes and a large region of AcNPV sequence flanking the cloning region to facilitate homologous recombination.

The pAcSecG2T transfer vector used in this study is a modification of the AcNPV vector. In addition to the above components, the pAcSecG2T vector contains a *GST*-coding sequence upstream of the gene cloning region, facilitating the expression of the required gene as a GOI-GST fusion protein from the recombinant Baulovirus. The vector also contains the *gp67* secretion signal to facilitate secretion of the produced protein out of the cells [Appendix 1].

Production of a recombinant virus involves the cloning of the *GOI* into a transfer vector, downstream of a polyhedrin promoter and co-transfecting the recombinant transfer vector and the Baculoviral DNA into insect cells, where recombination occurs. This results in the ***transfer of the GOI and the polyhedrin promoter into the virus replacing the virus's polyhedrin gene, producing a recombinant Baculovirus.*** (Figure 12 B) If the transfer vector is the pAcSecG2T vector, the gp67-GST-GOI region is transferred to the Baculovirus.

The recombinant Baculovirus produces the recombinant protein under the cell's expression system and infects other host cells. The recombinant virus can be identified as occlusion body negative plaques.



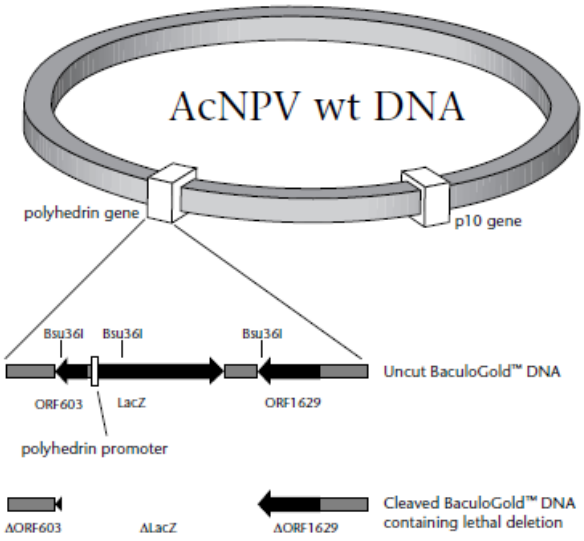
BD BioSciences. BEVS Manual. 6th Ed. 1999

Figure 12: Life cycle of the Baculovirus. (A) *In vivo*. The infection of host cells produce two distinct viral populations, occluded and non-occluded. When infected, the virion migrates to the nucleus where it sheds the capsid and proliferates. Secondary infection takes place when the

budded form enters new host cells. (B) *In vitro*. The Baculovirus DNA and the GOI containing recombinant transfer vector are co-transfected into the insect cells, where homologous recombination occurs. The recombinant virus can express the recombinant protein as well as infect new host cells (BD BioSciences. Baculovirus Expression Vector System Manual, 6th Ed. 1999).

1.5.5.1. BaculoGold™ linearized Baculovirus DNA

This modified form of the AcNPV viral DNA does not code for a viable virus as it contains a lethal deletion spanning 1.7Kb downstream of the polyhedrin gene (Figure 13). This deletion is rescued only by homologous recombination with a co-transfected transfer vector. Therefore, recombination frequencies exceed 99%.



BD BioSciences. BEVS Manual. 6th Ed. 1999

Figure 13: AcNPV BaculoGold™ DNA. The polyhedrin locus of the virus has been modified by replacing the polyhedrin gene with a LacZ gene and by addition of three Bsu36I cut sites. The DNA is linearized at the cut sites.

1.5.6. GST purification and processing of fusion proteins

The purification method is based on the affinity of the GST tag on a fusion protein to a Glutathione-coupled resin (Smith et al., 1988). The protein of interest is fused to GST, with an enzyme cut site (e.g. Thrombin cut site) between to cut and purify the fusion protein from the GST tag. The fusion protein containing solution is sent over a Glutathione coupled resin, which binds the GST component and thereby the fusion protein. The column is then washed with reduced Glutathione, which competes with the protein to bind to the column. Consequently,

the protein is released into solution. The purified fusion protein is then treated with Thrombin enzyme to cleave off the GST tag.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II: MATERIALS AND METHODS

2.1. JHAMT knockdown and its role on courtship behavior

2.1.1. Fly stocks

Drosophila melanogaster strains used-

Drosophila Stock	**Provider**
X/Y; +/+; hsp70-GAL4/+	Courtesy of Dr. Gregg Roman
X/Y; UAS- JHAMT-RNAi/ UAS- JHAMT-RNAi; +/+	Vienna *Drosophila* RNAi Centre
yw/Y; UAS-HID ala 5/ Cyo; Dno 43	Courtesy of Dr. A. Bergman
X/Y; +/+; to^1 2541/to^1 2541	Boomington stock 2541. (Dauwalder et al., 2002)
X/Y; $Gal80^{ts}$/Cy; repoGAL4/TM_3Sb	Courtesy of Dr. Gregg Roman
X/Y; +/+; UAS-LacZ/UAS-LacZ	Courtesy of Dr. Gregg Roman
X/Y; +/+; UAS-DTI/TM_3Sb	Courtesy of Dr. Gregg Roman
UAS-dicer/UAS-dicer; +/+; +/+	Courtesy of Dr. Gregg Roman
Canton-S (CS) (Wild type)	Courtesy of Dr. William Mattox
w^{1118}/Y; Cy/Sco; TM_3Sb/TM_2	Courtesy of Dr. Gregg Roman
w^{1118} Canton-S (w^{118} CS)	Courtesy of Dr. Gregg Roman

Table 1- Fly stocks that were used for the creation of genetic mutants used in the study and their sources

All flies were maintained on standard cornmeal/ sugar-based medium at room temperature.

2.1.2. Creating the *JHAMT-GAL4* fly line

The Corpora allata specific *GAL4* fly line was created to specifically target the organ using RNAi. To achieve this, a 5043 bp promoter region upstream of the translation start of *JHAMT* (CG 17330) gene [Appendix 1 A] was amplified by PCR and cloned upstream of the *GAL4* sequence into the Not1 and BamH1 restriction sites of the *GAL4* vector, pPTGAL.

Not1 and BamH1 restriction enzyme sites were added to the upstream and downstream primers, respectively.

Primer sequences used to amplify the region are as follows=

1. 5' end primer with Not1 site (*JHAMT* promoter s-3)-

5'-ATA AGA ATG CGG CCG CTG CGG TTT AGG GGT GCT ATG ACT-3'

2. 3' end primer with BamH1 site (*JHAMT* promoter as-4)-

5'- GCG GAT CCC TCG ACA ACT GAT CGA CGA TTG GGA C- 3'

Yellow- sequence of end of amplified promoter region

Red- Restriction enzyme sequence

Blue- Extra sequences

Genomic DNA was extracted from CS whole flies using the DNAzol (DNAZol® Reagent. Genomic DNA Isolation Reagent) DNA extraction method (Invitrogen). The DNA was used to perform a

gradient two-step PCR reaction using the above primers. Phusion DNA polymerase (Finzymes. Thermo Scientific) was used to amplify the sequence.

Conditions used for PCR amplification=

Initial denaturation	98°C	30 sec	
Denaturation	98°C	30 sec	
Annealing	57.1°C	15 sec	(6 cycles)
Extention	72°C	2 min	
Denaturation	98°C	10 sec	
Annealing	62°C	15 sec	(24 cycles)
Extension	72°C	2 min	
Final extension	72°C	10 min	
Hold	4° C		

The annealing temperatures indicated for the two reactions produced the best PCR product.

The amplified region of 5043 bps was cloned into the multi-cloning site of the PSC-B vector (Stratagene®) [Appendix 1 C].

The pSC-B vector is 4.3 Kb in size. The resulting construct of pSC-B and *JHAMT* promoter region was 9.5 kb in size. Correct clones were identified by restriction digest and DNA was prepared by QIAGEN plasmid midi-prep.

The insert was released from pSC-B and cloned into the Multi cloning site of a pPTGAL *GAL4* vector, using the restriction enzymes Not 1 and BamH1 [Appendix 1 D & E].

The construct region was sequenced (SeqWrite Inc.) to verify the sequence. The sequencing primers used are as follows:

1. *JHAMT* Seq 3'- 5'- GGA GAG TGG GTT TTC TGG CGA GAG-3'

2. *JHAMT* Seq 5'- 5'- ACA TAG CAT AAG TTT CGA TTA AGC CT-3'

Plasmid DNA was prepared using the QIAGEN plasmid midi-prep Kit.

The pPTGAL4-*JHAMT* was injected into w^{1118} CS embryos by Rainbow Transgenic Flies, Inc., and transgenic lines established.

Creating the *JHAMT-GAL4* transgenic flies

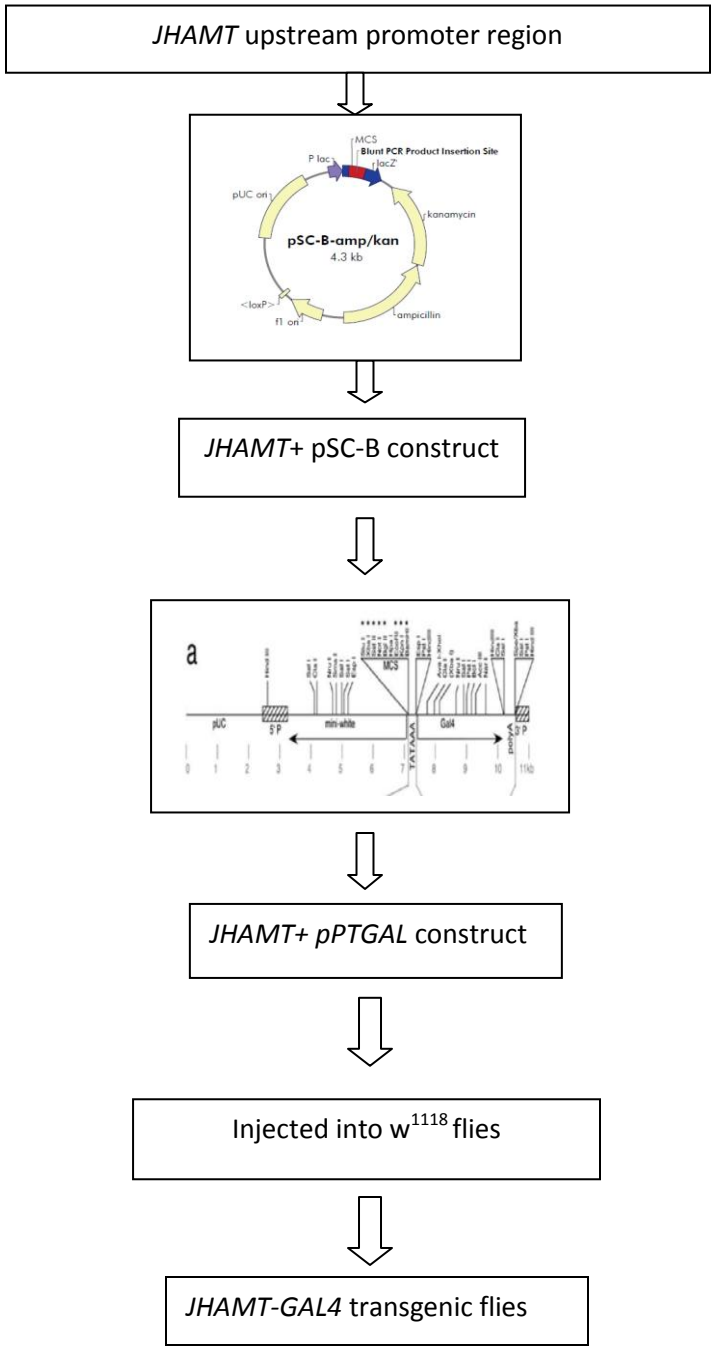


Figure 14: Flow diagram of creation of *JHAMT-GAL4* flies. *JHAMT* upstream promoter region was amplified from genomic DNA using PCR. The sequence was cloned into an intermediate pSC-B vector and sequence verified. The sequence was then cloned from pSC-B into a pPTGAL vector upstream of the *GAL4* sequence. The pPTGAL+ *JHAMT* construct was injected into w^{1118} flies.

2.1.3. Mapping the fly lines

Individual transgenic fly lines were mapped to locate the chromosome containing the *JHAMT-GAL4* construct. The transgenic flies emerging from the embryos after injection of the construct were crossed to w^{1118} *CS* flies. The progeny were screened for presence of non-white eye color. Orange-red eye color indicates the presence of the w^{+} marker indicating the presence of the construct. w+ flies were crossed to w^{1118}*/Y; Cy/Sco; TM3,Sb/TM2* double balancer and subsequently to w^{1118} flies to locate the chromosomes containing the construct. 14 fly lines were mapped.

2.1.4. Screening of fly lines

The transgenic fly lines were screened for expression of the *JHAMT-GAL4* construct in the Corpora allata to select a line showing optimal expression in the organ. This fly line is to be used in further studies. To achieve this, each of the *JHAMT-GAL4* transgenic lines was used to drive the expression of a *UAS-LacZ* construct placed on the 3rd chromosome. The transgenic flies contained the *JHAMT-GAL4* construct in either the X, 2nd or 3rd chromosome.

The flies were freeze-sectioned to 20µ thick sections at -20 °C using a Microm HV 560 cryostat. The sections were dried at room temperature for several hours and fixed in fixative solution. (0.1 M $Na_3(PO_4)$ 600µl, 1mM $MgCl_2$ 6µl, 1% Glutaraldehyde 240µl, and 5.154 ml H_2O) for 15 min. They were then submerged in (0.1M Phosphate buffer $Na_3(PO_4)$) and stained with X-Gal (2% in DMSO) for 15 minutes. The staining reaction was halted by transferring to TE (10 mM Tris, 1mM EDTA) solution.

The staining solution contained the following-

1. 30µl 1M $Na_3(PO_4)$ pH 6.9
2. 90µl 5M NaCl
3. 3 µl 1M $MgCl_2$
4. 100 µl 100mM $KFe(CN)_6$.$3H_2O$
5. 100 µl 100mM $KFe(CN)_6$
6. 300 µl 2% X-Gal in DMF or DMSO
7. 2.4 ml distilled H_2O

The sections were observed using an Olympus, BX60 microscope.

2.1.5. Verification of *JHAMT-GAL4* expression in the Corpora allata using immunohistochemistry

The flies were selected and matured for 4 days and embedded in OCT (Optimal Cutting Temperature Medium). The flies were then freeze-sectioned at -20°C using a Microm HV 560 cryostat. The sections were fixed in 4% paraformaldehyde in PBSH (PBS/ Phosphate Buffered Saline containing 1M NaCl) for 20 minutes at room temperature. All subsequent procedures were performed at room temperature except antibody incubations, which were done in at 4°C. Sections were washed thrice in 1X PBSH/ 0.5% Triton X-100 for 15 minutes and washed thrice in TNT buffer (0.1M Tris-Cl/ 0.3M NaCl (pH 7.4), containing 0.5% Triton X-100) for 15 minutes each. Sections were then blocked in 4% normal goat serum in TNT (blocking buffer) for 1 ½ hours.

Double staining using α-β-Gal and α-JHAMT antibodies was performed on the sections. The α-JHAMT antibody raised in rabbit was a kind gift of Ryusuke Niwa, (Univ of University of Tsukuba, Japan; Niwa et al., 2008)and was used at a 2:200 concentration. The secondary antibody was Alexa 546 conjugated α-rabbit antibody at a 1:200 concentration (Invitrogen). Mouse α-β-gal antibodies (Sigma) were used at 1:200. The secondary antibody was Alexa 633 conjugated α-mouse antibody raised in goat (Invitrogen), applied at a 1:200 concentration.

All antibodies were diluted in 4% normal goat serum in blocking buffer.

The primary antibody incubations were done overnight at 4°C. The sections were washed 6 times for 15 minutes each and 5 times for 30 minutes each in TNT. Secondary antibody incubations were done overnight at 4°C. The sections were washed 5 times for 15 minutes each in TNT.

The fluorescently labeled sections were mounted in DAPI containing Vectashield mounting medium (Vector Laboratories. CA).

Confocal microscopy

Fluorescent preparations were viewed using an Olympus FV 1000 Confocal microscope.

2.1.6. Knockdown of *JHAMT* at room temperature

The above created *JHAMT-GAL4* driver line was used to knockdown JHAMT protein levels in the Corpora allata, using a *UAS-JHAMT-RNAi* construct at room temperature. A *UAS-dicer* construct was also used to enhance knockdown effect.

The flies were raised at room temperature. The selected virgin males were isolated for 4 days and subjected to a courtship assay. The flies of experimental and control genotypes were also reared and matured as for the courtship assay and were subjected to a short term activity assay.

2.1.7. Conditional knockdown of *JHAMT* in adult flies using the *GAL80^{ts} system*

The *Gal80^{ts}* system was used to drive the RNAi construct (*UAS-JHAMT-RNAi*) and cell death constructs (*UAS-HID* and *UAS-DTI*) conditionally in adults. The flies were raised at 18°C. The selected flies from each genotype were maintained at 18°C for 8 days until maturity. They were then induced at 32°C for 2 days and rested for 1 hour prior to courtship assay. Activity assays were performed.

2.1.8. Conditional knockdown of *JHAMT* in adult flies using the *hsp70-GAL4* system

hsp70-GAL4 was used to drive the expression of the *UAS-JHAMT-RNAi* construct conditionally in adults. The flies were raised at room temperature and the selected flies were isolated for 4 days. The *hsp70-GAL4* driver was induced by placing the flies in pre-heated vials at 37°C for 1 hour in an incubator. The flies were then transferred to fresh vials and placed at room temperature for

4 hours to rest. They were then subjected to a courtship assay. Activity assays were performed for flies of the same genotype.

2.1.9. Methoprene treatment of flies

0.5% Methoprene was prepared by dissolving Methoprene (ChemService) in 100% Acetone. The flies were anaesthetized on ice and treated with the Methoprene solution (1µ of solution was applied on the ventral surface of the abdomen using a micropipette). The control flies were treated with Acetone only. The Methoprene treated and untreated (Acetone treated) flies were placed for 4 hours at room temperature and subjected to a courtship assay.

2.1.10. Courtship assay and short term activity assay

2.1.10.1. Courtship assay. The selected matured flies of the experiments were placed individually in a mating wheel with a 2-3 hours old immature virgin Canton-S (CS) female. Courtship was observed for a period of 10 minutes (600 seconds) to record the fraction of time any/all steps of courtship (orientation, tapping, singing, attempted copulation) were performed. The courtship index was recorded.

Courtship Index (CI) = $\frac{\text{Period of time (in seconds) male engages in courtship}}{600}$

Courtship index was plotted against the genotype.

2.1.10.2. Short term activity assay. Flies of genotypes used for courtship assay were tested for locomotion defects. Flies were selected and matured as for courtship assays. They were placed individually in mating wheels on a filter paper marked with a horizontal line. The fly was given an acclimatization period of 2 minutes. The number of times a fly crosses the line within the next 3 minute period was recorded.

Activity Index (AI) = Number of times fly crosses the line within a 3 minute period

Activity index was plotted against the genotype.

2.1.11. Statistical analysis

Data are presented as means $\pm$ standard error of the mean (s.e.m). Statistical comparisons between the genotypes were performed by one-way ANOVA or two-way ANOVA with Bonferroni multiple comparison Post-hoc test. Statistical analysis was performed using the StatView® statistical analysis program.

2.1.12. Verification of *JHAMT* knockdown in RNAi experiments

2.1.12.1. Dissection of ovaries

Drosophila females from the genotypes used for behavior assays were dissected 3 days after eclosion. The females anesthetized using CO_2, were placed in 1X PBS and the abdomens were dissected open to reveal the ovaries.

2.1.12.2. Western blotting (Immunoblotting)

Western blotting was performed on whole fly protein extracts.

Flies were frozen on dry ice and placed at -80°C until protein extraction. 50 flies were used per genotype. The flies were homogenized in EB-2 buffer (0.1M HEMG, 5mM EDTA, 1mM $MgCl_2$, 1 mM DTT, and 10X Triton-X-100) containing a protease inhibitor cocktail (Roche Complete Mini. 04693124001) (0.1 M HEMG: 100 mM KCl, 20 mM Hepes pH 7.6, 5% Glycerol).

Flies were homogenized in 3 volumes of cold EB-2 buffer containing protease inhibitor cocktail (857 µl buffer + 143 µl inhibitor) using Kontes tubes and pestles. The homogenate was centrifuged at 16,000 g for 15 min. Homogenate was transferred to new tubes and centrifuged again for 15 min. as above. The supernatant was transferred to a new tube and protein concentration measured using a BioRad Lowry based assay (BioRad. D_c Protein Assay. Reagent A and Reagent B). 0.1 OD of each sample was loaded onto a gel.

Protein extracts were run on a 12% polyacrylamide mini-gel along with a Fermentas® pre-stained (Fermentas®. PageRuler™ Plus pre-stained protein ladder) marker. 10µl of protein was mixed with Laemmli buffer, boiled, chilled on ice, and loaded onto the gel.

The protein samples and marker were transferred onto a PVDF membrane (BioRad. Immunoblot PVDF membrane) at 4°C for 90 minutes in blotting buffer (0.048M Tris, 0.039M Glycine, 200 ml Methanol, 0.001M SDS, 800 ml distilled water). The blots were blocked for 1 ½ hours in 5% blocking solution (GE Healthcare®, Amersham™. ECL Prime Blocking Agent. RPN 418) and incubated with the primary antibody (rabbit-raised α-JHAMT) at a 4:5000 concentration in blocking buffer overnight. The blots were washed in TBST solution (100 ml 10X TBS (1.4M NaCl, 0.1M Tris-Cl pH7.5, 1800 ml distilled water, 2 ml Tween-20). The blots were washed in TBST 3 times for 5 minutes each and 2 times for 30 minutes each and once for 3 ½ hours. The blot was subsequently incubated with Horse Radish Peroxidase (HRP) conjugated secondary antibody, α-rabbit at 1:10,000 dilution (Jackson Immunoresearch laboratories Inc.). The blots were washed in TBST 3 times for 10 minutes each and 2 times for 20 minutes and once overnight. The overnight wash was done at 4°C. The antibody was visualized using the Enhanced Chemiluminescent detection kit (GE Healthcare®, Amersham™. ECL Select™. Western Blotting Detection Reagent). The blots were exposed to UltraCruz® blue autoradiography film (Santacruz Biotech).

2.1.12.3. qPCR (Quantitative Reverse Transcription Polymerase Chain Reaction)

2.1.12.3.1. RNA extraction and reverse transcription

RNA extraction was performed on 5-8 flies per genotype using the Trizol (Invitrogen) reagent. The selected flies were homogenized in 1 ml of Trizol reagent and kept at room temperature for 5 min. They were then centrifuged at 12,000 g for 10 min. The cleared homogenate was mixed vigorously with 0.5 ml chloroform, incubated at room temperature for 3 minutes and centrifuged at 12,000 g for 15 min. The aqueous phase contained the RNA. The RNA was

precipitated by transferring to a fresh centrifuge tube and mixing with 0.5 ml isopropanol. After 15 minutes incubation at room temperature, samples were centrifuged at 12,000g for 10 min. The supernatant was removed and the RNA pellet was washed in 1 ml of 75% ethanol (in DEPC/ Diethylpyrocarbonate treated water) and centrifuged at 7500g for 5 minutes. The ethanol was removed, the pellet air dried and re-dissolved in 30µl of DEPC water. The RNA in DEPC water was stored at -80°C. The RNA was quantified using a spectrophotometer (NanoDrop. ND-1000. Spectrophotometer).

cDNA synthesis of each sample was carried out using 4 µg of total RNA using Superscript II (Invitrogen. Superscript-II-Reverse Transcriptase). The RNA was DNAse treated in DNAse buffer (Promega), DNAse (Promega) and DEPC-treated water in a 20µl volume by incubating at 37°C for 90 min. DNAse treatment was stopped by adding 1µl of DNAse stop solution (Promega) and heating to 65°C for 10 min. Samples were transferred to 4°C. cDNA synthesis was done by mixing 10µl of DNAse treated RNA with 2µl of oligodT (12-18) (Invitrogen), 2µl of 10mM dNTP mix and DEPC water. The mix was incubated at 65°C for 5 min and quickly chilled on ice. After centrifuging the contents, each sample was divided to two, as RT+ and RT-. Each sample (12µl) was mixed with 4µl of 5X first strand buffer, 2µl of 0.1M DTT (Invitrogen) and 1µl of RNAseIn Ribonuclease Inhibitor (Promega) and incubated at 42°C for 2 min. Subsequently, the RT+ sample of each RNA sample was mixed with 1µl of SuperScript RT II. The RT- sample was mixed with 1µl of DEPC water. All samples were incubated at 42°C for 50 min for the reverse transcription reaction. The reaction was inactivated by heating at 70°C for 15 min. The prepared cDNA was stored at -80°C.

2.1.12.3.2. Quantitative PCR (qPCR)

TaqMan probes for *JHAMT* and *rp49* were used following the company protocol. (AppliedBiosystems) (http://www.lifetechnologies.com/global/en/website-overview/ab-welcome.html).

TaqMan Gene Expression Assay

JHAMT probe- TaqMan assay ID= Dm01791790_gl. Size FAMS: 360 rxns. Cat. 4351372

rp49 probe- TaqMan assay ID= Dm02151827_gl. Size FAM: XS 75 rxns. Cat. 4453320

2.1.12.3.3. Establishing a standard curve and qpcr assay

A standard curve was established for wildtype CS cDNA, to determine the quantity of RNA of the experimental and control genotypes to use for quantification. Dilutions of 1, 0.1, 0.01, 0.001, and 0.0001 of the RT+ and RT- cDNA were used.

The qPCR assay for each sample was performed in a 20 µl mix consisting of 10µl TaqMan Master mix (Applied Biosystems), 2µl cDNA, 1µl TaqMan probe and DEPC treated water. The samples were loaded in triplicate into a 96 well plate. The amplification was performed for 40 cycles in an Applied Biosystems 7000 qPCR machine.

The undiluted RT+ cDNA preparations of experimental samples were loaded in triplicate into the 96-well qPCR plate. The previously prepared CS cDNA was used for a wildtype control and as a standard between assay plates. 50 cycles were performed.

2.1.12.3.4. Data analysis

Analysis and relative expressions were determined using the Comparative Threshold Cycle (ΔΔCt) method. The GOI (Gene of Interest) was *JHAMT* while the reference was rp49 (Ribosomal Protein49). The change in mRNA expression was calculated as fold change relative to control. The ΔC_t (normalized C_t) value was calculated as the difference between the C_t (threshold cycle value) for the expression of *JHAMT* and the endogenous control, rp49 for each genotype. The calibrated ΔC_t value for each sample ($\Delta\Delta C_t$) was calculated as the difference between the ΔC_t values of the sample and the wildtype control CS. The fold change for each sample relative to the calibrator was calculated as $2^{-\Delta\Delta Ct}$. The fold induction values for each genotype are plotted as a bar graph (Bookout and Mangelsdorf, 2003).

A calculation template kindly provided by Dr. Cecilia Williams was used to analyze the data.

2.2. Binding between Takeout and Juvenile hormone

The Takeout protein was expressed as a fusion protein using the BEVS. The *takeout* sequence amplified from *takeout* cDNA was cloned downstream of a *GST* sequence into a pAcSecG2T transfer vector. The total *takeout-GST* sequence was positioned downstream of a polyhedrin promoter. The transfer vector carrying the *takeout*-GST sequence was co-transfected with BaculoGold™ Baculovirs DNA (BD BioSciences. PharminGen) into Sf9 cells in serum containing culture medium. The resulting recombinant virus was amplified and used for protein expression. The protein expression was carried out by re-transfecting the Sf9 cells with the recombinant virus and maintaining the cultures at 28°C. The resulting protein was subsequently harvested from the supernatant/ culture medium, purified from the medium using GST purification and processed by enzymatic cleavage using Thrombin. The cleaved protein was used for the binding assay between takeout and Juvenile hormone.

Tritium labeled Juvenile hormone (JH^{H3}) was used to examine the binding between Takeout protein and Juvenile hormone in an equilibrium dialysis assay using JH^{H3}.

2.2.1. Creation of the recombinant BaculoGold-*takeout-GST* virus

2.2.1.1. Creating the pAcSecG2T-*takeout* construct

The sequence of *takeout* was amplified from *takeout* cDNA open reading frame using PCR amplification (Figure 15) [Appendix 2 A and B]. The open reading frame (758 bps) excluded the initial signal peptide region (54 bps) of the *takeout* sequence. The signal peptide region was removed as the transfer vector that was used to transfer the *takeout* gene sequence to the

BaculoGold™ DNA by recombination (pAcSecG2T vector) includes a secretion signal upstream of the insertion site to facilitate secretion out of the cells into the culture medium.

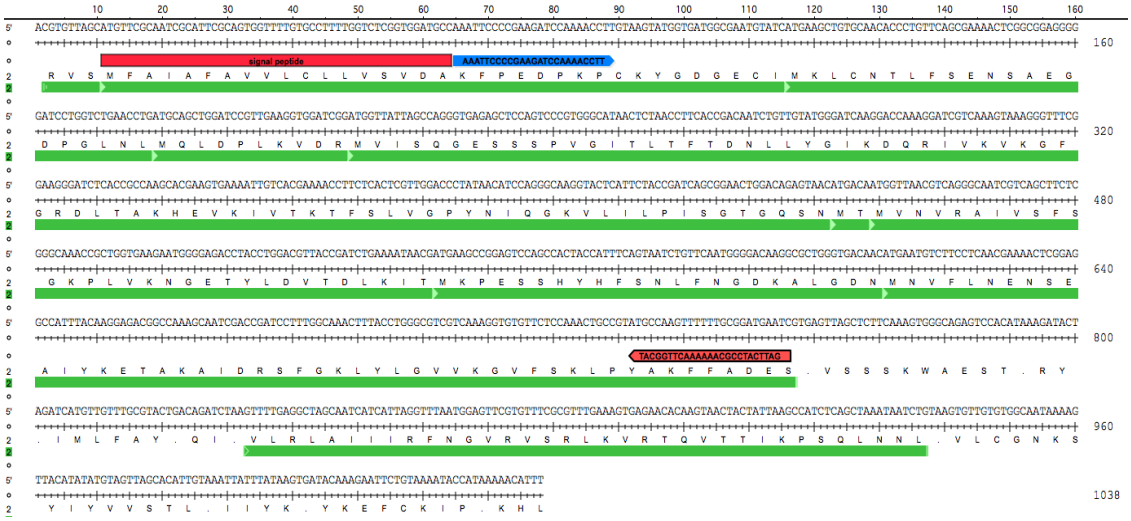


Figure 15: The *takeout* sequence indicating the regions used for PCR primers.

Sma-1 restriction enzyme site sequences were added to the PCR primer sequences, as the Sma-1 restriction enzyme will be used to clone the *takeout* insert into the Baculovirus transfer vector (pAcSecG2T) (BD BioSciences. Pharmingen). Extra sequences were added for the efficient cleavage by Sma-1. Sequences of the PCR primers used to amplify the region are as follows;

5'- TCC ***CCC GGG*** AAA ATT CCC CGA AGA TCC AAA ACC TT- 3'

5'- TCC ***CCC GGG*** GGT CAC GAT TCA TCC GCA AAA AAC TTG-3'

Yellow- sequence of end region of amplified *takeout* sequence

Red- Restriction enzyme region

Blue- Extra region

The cDNA of *takeout* was amplified using Phusion High Fidelity polymerase (Finzymes). A PCR amplification program with two step gradients was used.

Conditions used for PCR amplification:

Initial denaturation	98°C	30 sec		
Denaturation	98°C	30 sec	15 sec	(X6)
Annealing	56.8 (clone 1) and 65.2 (clone 6) °C			
Extention	72°C	2 min		
Denaturation	98°C	10 sec	15 sec	(X24)
Annealing	61.8 (clone 1) and 67.2 (clone 6) °C			
Extension	72°C	2 min		
Final extension	72°C	10 min		
Hold	4° C			

The two-step PCR reaction was done for 9 samples. The annealing temperatures had a range of (56-72)°C for the first step and a range of (61-72)°C for the second step. The annealing

temperatures indicated in the schematic above, refer to those used for the two clones that proceeded into mini-prep.

The resulting PCR products were run in a 1% agarose gel at 68 V for 60 min and stained using ethidium bromide.

The *takeout* sequence was cloned into the Multi Cloning Site of a pSC-B vector (StrataClone. Agilent Technologies). This vector was used as an intermediate vector for sequence verification. [Appendix 2 C] Cloning was done according to manufacturer's protocol (StrataClone. Agilent Technologies). This resulted in a 5.058 Kb pSC-B + *takeout* construct.

'pSC-B + *takeout*' constructs amplified by QIAGEN mini- prep and were run in a 1% agarose gel.

Two of the obtained constructs, clone 1 and clone 6 were tested for the presence of the *takeout* sequence by restriction enzyme digestion using Sma-1 restriction enzyme.

Clone 1 and clone 6 sequenced for further verification of the presence of the *takeout* sequence (SeqWrite Inc.). The sequences primers used for sequencing are as follows;

M13 forward primer- **5' GTTGTAAAACGACGGCCAGT 3'**

M13 reverse primer- **5' CACAGGAAACAGCTATGACC 3'**

The *takeout* sequence was cloned from the pSC-B + *takeout* construct into the pAcSecG2T vector. The recipient vector (pAcSecG2T) and PSC-B + *takeout* construct were digested to clone the *takeout* sequence into pAcSecG2T. The restriction enzyme used was Xma-1 (New England

Biolabs). Xma-1 is an isoschizomer of Sma-1. Therefore, it recognizes the restriction enzyme sequence of Sma-1 and cuts at a different cut site.

Sma-1 restriction enzyme recognition sequence;

5’-CCC↓GGG-3’

3’-GGG↑CCC-5’

Xma-1 restriction enzyme recognition sequence;

5’- C↓CCGGG-3’

3’-GGGCCC↑-5’

(Arrows indicate cut sites)

The presence of the *takeout* insert in the recombinant vector was verified by an Xma-1 restriction enzyme digest.

A BamH1 (New England Biolabs) digestion was carried out to verify the orientation of the insert.

The correct orientation of the insert should result in a 90 bp fragment of DNA along with an 8551 bp band.

The constructs were then sequenced for verification

The sequencing primers used were,

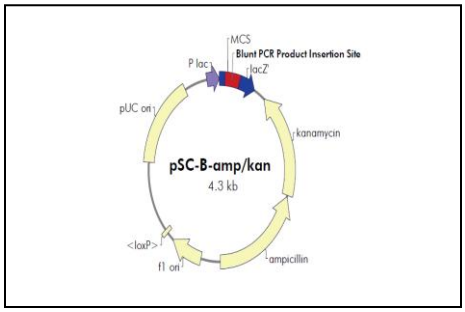
1. D12 as-9 5’- TACATTCGCCATCACCATACTTAC-3’

2. D 12 as/5 5’- CTTGGCATACGGCAGTTTGGA-3’
3. D12 s2 5’- TCCAAACTGCCGTATGCCAAG-3’
4. D12/s -1 5’- ACCTTCTCACTCGTTGGA-3’
5. Takeout s-20 5’- GACAAGGCGCTGGGTGACAAC- 3’

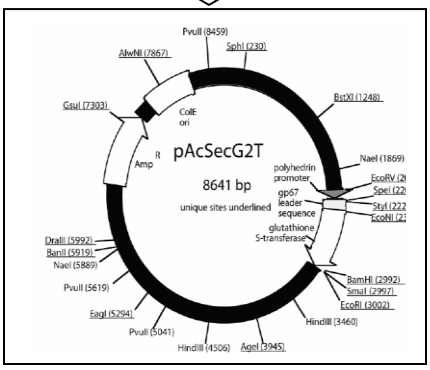
[Appendix 2 D]

Creation of the BD BaculoGold-*takeout-GST* recombinant virus

takeout sequence (without secretion signal)



takeout+ pSC-B construct



takeout + pAcSecG2T construct

BD BaculoGold Baculovirus

Recombinant BD BaculoGold-*takeout-GST* virus

Figure 16: Creation of the BD BaculoGold-*takeout-GST* recombinant virus. Flow diagram of the creation of the recombinant virus. The *takeout* sequence amplified from cDNA was cloned into a pSC-B intermediate vector and subjected to sequence verification. The *takeout* sequence was subsequently cloned from the pSC-B vector into the pAcSecG2T Baculovirus transfer vector and finally subjected to *in vitro* recombination along with the *GST* sequence of the transfer vector with the BD BaculoGold™ virus in Sf9 cells to obtain the BaculoGold-*takeout-GST* recombinant virus.

2.2.1.2. Recombination between pAcSecG2T+*takeout* vector and BD BaculoGold DNA

The Baculogold-*takeout-GST* virus was created *in vitro* using Spodoptera frugiperda (Sf9) insect cells. The prepared construct and the virus were transformed into Sf9 cells. BD Baculogold expression vector system was used for this purpose. The steps of this process are as follows-

1. Establishment of Sf9 cells in serum containing TNM-FH medium
2. Co- transfection of the construct and BD BaculoGold™ virus and *in vitro* recombination
3. Harvesting of the virus and storage

Live Sf9 cells (BD Biosciences) cells were cultured in TNM-FH medium (BD BioSciences) containing 10% FBS. Gentamicin (BD BioSciences. Pharmingen) was added at a 50 µg/µl concentration as an antibiotic. Cells were seeded at a density of 4 x 10^6 and 7 x 10^6 cells/ml. Cell counts and viability were tested using a hemocytometer and Trypan Blue exclusion assay. Cultures were placed as monolayer cultures and maintained in T75 flasks inside a 28°C incubator. Cultures were split 1:3 when cells became confluent.

The BD BioSciences expression vector system manufacturer protocol (BD BioSciences) was followed for the recombination between pAcSecG2T + *takeout* and BD BaculoGold Virus.

The pAcSecG2T vector containing the *takeout* sequence and the BaculoGold linearized DNA were co- transfected into Sf9 cells cultured in TNM-FH medium. As a transfection control, the empty vector pAcSecG2T was used. The quantities of each that were used for transfection are as follows:

1. BD BaculoGold linearized DNA 0.5µg
2. pAcSecG2T vector or pAcSecG2T +*takeout* construct 2µg

[The pAcSecG2T vector without the *takeout* sequence was used to recombine into the BD BaculoGold virus to act as a transfection control. This virus will express the GST protein. The BaculoGold + *takeout-GST* virus will express the 'Takeout-GST' protein. It was to be used to clearly identify the 'Takeout-GST' protein band of experimental construct expression from all other possible protein bands. The control virus can also be used to test if recombination occurs between the virus and the construct.]

The cells were seeded to a 70% confluence and 2 x 10^6 cell density in a 60 mm tissue culture plate. After the cells were attached to the plate, the TNM-FH medium was replaced by 1 ml of Transfection buffer A (BD Biosciences).

The pAcSecG2T vector or pAcSecG2T -*takeout* construct and BaculoGold™ DNA were mixed and 1 ml of transfection buffer B (BD Biosciences) added. The transfection buffer B/ DNA solution was added drop wise into the plated Sf9 cells. The plate was rocked while the transfection mix was being added. The plate was incubated for 4 hrs at 27°C and subsequently, the medium was changed to TNM-FH medium and incubated in the above conditions for 4 days.

After 4 days, the supernatant was removed and used to infect more cells for amplification.

The recombinant viruses (experimental and control) were amplified to obtain a high titer stock solution. Insect cells were freshly seeded to a density of 5 x 10^6 cells in 15 ml of TNM-FH medium in a per 10 cm plate (60% confluent). 500 µl of viral supernatant was added to the plate and incubated in a 27°C incubator for 3 days. The resulting supernatant was subjected to a second round of amplification. The viral harvest from the second amplification was subjected to a third round of amplification. The final amplified viral supernatant was harvested and stored. Harvesting and storage of the viruses was done as follows.

Virus was harvested from transfected Sf9 cell plates after 4 days. The cell plates were examined under a light microscope. Cell bursting and presence of cell debris indicates viral tranfection and discharge of the virus into the supernatant. The supernatant was aspirated and centrifuged for 5 min at 500g. The virus was stored at 4°C and protected from light.

2.2.2. Cryopreservation of cells

Cell samples were prepared to a density of 4x 10^6cells/ml. Centrifuge tubes containing the cell samples were centrifuges at 1000xg for 10 min. Supernatant was decanted and cell pellet kept on ice. The cell pellet was re-suspended in 90% TNM-FH + 10% DMSO (di-methyl-sulfoxide) and transferred to cryovials. The vials were placed in the cell freezing device and placed in a -80°C freezer.

2.2.3. Analysis of protein samples

The expression of *takeout-GST* was analyzed by PolyAcrylamide Gel Electrophoresis/ PAGE.

The protein samples were run in a SDS polyacrylamide gel alongside a pre-stained protein ladder (Fermentas®Page Ruler Pre-stained ladder).The gels were analyzed for protein expression using Coomassie staining and Immunostaining.

Preparation of cells: The pelleted cell were lysed by adding 100 µl of 1X Laemmli buffer (60mM Tris-Cl pH 6.8, 2% SDS, 10% Glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and vortexing for 10 seconds. The sample was boiled for 3 minutes and cooled in ice.

Coomassie staining was carried out to visualize the protein bands in the gels, mainly for quantification purposes, using a methanol-based Coomassie stain (Omnipur. EMD Chemicals). The samples were run in the protein gel alongside a BSA std. protein marker (BioRad) for quantification. The gels were washed in ddH_2O for 10 minutes. They were placed in Coomassie stain for 4 hours for staining and de-stained in ddH2O overnight. The washes, staining, and de-staining were done on a constantly rocking platform.

Western blotting was performed to examine for the presence of the desired proteins by staining them using antibodies raised against the protein. Specifically, α-GST (BD BaculoGold Purified Mouse Anti-Gutathione S-Transferase) antibody raised in mouse was used to detect the GST component of Takeout-GST fusion proteins and the GST control protein. An affinity purified α-Takeout antibody raised in rabbit was used to detect Takeout (Lazareva et al., 2007). As secondary antibodies, HRP conjugated α-mouse and α-rabbit antibodies raised in goat (Jackson Laboratories) were used, respectively. The detection reaction used an ECL/Enhanced Chemiluminescent detection kit (GE Healthcare). HRP is an enzyme that, in the presence of a substrate provided by the detection reagent, produces a detectable luminescent signal. The luminescent was detected by autoradiography (Santacruz Biotech.).

2.2.4. Protein expression in Sf9 cells in TNM-FH medium

For protein expression cells were grown to a 2 x 10^7 cells per plate density in 15 cm (T75) tissue culture plates. Fresh TNM-FH medium was added to make-up a total of 30 ml per plate.

Viral stock was used to infect the cells for protein expression. The cells were incubated for 3 days at 27°C. The cells and the supernatant were separated by centrifugation. The two fractions were stored at -20°C until analysis.

2.2.5. GST column purification of Takeout-GST protein

I used three different methods for GST purification

2.2.5.1. GST-purification mini-columns (Pierce Glutatione Spin Columns)

Pierce Glutatione Spin Columns® Themo Scientific. (0.2 ml resin bed) were used. 1 ml samples from supernatant of BaculoGold-*takeout-GST* (experimental) and BaculoGold-*GST* (control) cultures and an un-infected control culture were used for analysis following the manufacturer's protocol. Each 1ml protein sample was concentrated to 50µl using Millipore concentrators.

Briefly, the columns were equilibrated to room temperature from a storage temperature of 4°C. All purifications were performed at room temperature. The storage buffer of the glutathione column was removed by centrifuging. The column was equilibrated in the equilibration buffer. All centrifuging were performed at 700xg for 2 minutes. The protein sample was mixed with equilibration/wash buffer (50mM Tris, 150mM NaCl pH 8) at a ratio of 50µl concentrated protein: 350µl buffer. The protein sample in equilibration buffer was applied to the column and incubated for 60 min on a rocking platform at room temperature. The flow through was removed by centrifuging and saved for subsequent analysis. The column was washed in equilibration buffer twice by adding buffer and centrifuging. The washes were saved for subsequent analysis. The Glutathione Agarose column bound protein was eluted by adding

Elution buffer (50mM Tris, 150mM NaCl pH 8) and centrifuging. The elution step was repeated twice. The samples were stored at -20°C.

2.2.5.2. GST purification using the BD BioSciences purification kit (Column Purification method)

The volume of medium used was 10 ml. The manufacturer's protocol was followed. The whole procedure was performed at 4°C.

Briefly, the glutathione agarose beads were gently re-suspended and poured into a 1 ml syringe in which the outlet was plugged with glass wool. The syringe was placed upright using a clamp and stand. The beads were allowed to settle and the column was allowed to drain. The beads were washed with 1X PBS buffer and allowed to drain. The protein sample was applied to the column and allowed to pass through. The flow through fraction was saved for analysis. The column was washed with 1X PBS buffer and allowed to drain. Three bead volumes of reconstituted GST elution buffer (elution buffer + Glutathion to a concentration of 5mM Glutathione solution) was added to the column and allowed to pass through. The eluted fraction was collected. The eluted protein sample was dialyzed against 100 volumes of 50mM Tris-HCl (pH=8) at 4°C overnight. The dialysis buffer was changed after 2 hours.

The purified protein sample was analyzed for successful purification using PAGE and Coomassie staining. 200 µl of purified protein was concentrated using Millipore concentrators to 25µl. 1 ml of un-purified sample was concentrated to 150µl. Samples were mixed with Lammli buffer and prepared for PAGE. 10µl each of the protein samples and protein standard were analyzed.

2.2.5.3. GST purification using the BD BioSciences purification kit (Batch Purification method)

The batch purification procedure of BD BioSciences GST expression and purification kit was used for purification of the Takeout-GST protein. The batch purification protocol was modified slightly to alter the incubation time of the column with the protein sample. Briefly, the glutathione beads were re-suspended and a 500µl sample was obtained to a sterile tube and centrifuged at 500 x g for 5 minutes. The supernatant was aspirated. The beads were washed with PBS buffer and centrifuged after each wash. The protein sample was mixed with the beads and incubated for 30 minutes at 4°C on a rocking platform. The slurry was centrifuged and the supernatant removed (the supernatant was analyzed for presence of protein). The fusion protein bound matrix was washed with 1X PBS buffer. Reconstituted GST elution buffer (containing Glutatione powder) as added to the bead matrix, gently mixed and incubated for *3 hours and overnight* (*in two separate experiments*) at room temperature. The sample was centrifuged to collect the elution fraction. The elution step was repeated twice.

The resulting elution fractions were pooled and concentrated. The flow from each experiment was also concentrated. The samples were mixed with Lamelii buffer, boiled and subjected to PAGE. The gels were subjected to Coomassie staining and Immunostaining.

2.2.6 Protein expression from the recombinant virus by Allele Biotechnology

The protein expression was not sufficient to obtain enough Takeout-GST protein to process, purify and perform binding assays. Therefore, the viral supernatant was sent to Allele Biotechnology to express the protein in mass scale. The viral stock of recombinant Baculovirus-

takeout-GST was amplified to obtain a high titer viral stock. The company then infected three different cell lines, Sf9 (grown in medium-containing serum), Sf9 super, and Tni (both grown in serum-free medium) in a pilot experiment. The cell pellets and 30ml each of supernatants were sent to us for analysis. I aliquoted the supernatants into 1ml samples. The cell pellets were suspended in 1ml of EB-2 buffer containing protease inhibitors. The cell pellets and supernatants were stored at -80°C.

2.2.7. Optimization of GST purification of Takeout-GST fusion protein

2.2.7.1. GST purification of Takeout-GST fusion protein after dialysis into 1X PBS buffer

The fusion protein was dialyzed into 1X PBS buffer overnight to purify the protein medium off sugars, peptides and other contaminants prior to GST purification. It was expected that this would enhance the binding of the protein to the Glutatione agarose bead column. The GST column purification was carried out on 10 ml of fusion protein in Sf9 super cell medium. The purification flow through was concentrated for subsequent analysis. The eluted protein was dialyzed overnight into 50mM Tris-Cl buffer. It was then concentrated. The concentrated protein sample and flow through was subjected to PAGE . The gel was coomassie stained.

2.2.7.2. Ammonium Sulphate $(NH_4)_2SO_4$ purification of Takeout-GST

The Takeout-GST protein was purified using $(NH_4)_2SO_4$ precipitation to enhance its binding to the Glutathione agarose column (by removing potential components in the medium that could interfere with binding to the column).

Purification of the protein using $(NH_4)_2SO_4$ to examine the efficiency of the procedure: 3 ml of the protein expressed in Tni medium was prepared. $(NH_4)_2SO_4$ was added to 80% saturation (1.7g $(NH_4)_2SO_4$ to 3ml Tni medium). The $(NH_4)_2SO_4$ was added gradually, under constant stirring at 4°C. The stirring was continued for 1 hr. The solution was centrifuged for 15 minutes at 15,000g at 25°C. A protein pellet was not visible. The $(NH_4)_2SO_4$ treated sample was analyzed for the presence of the protein. Three regions of the sample (top area surface, solution and pellet area) were analyzed by antibody staining using α-GST antibody. 10µl samples from the top and center areas were analyzed. The pellet area of the tube was washed using 10µl of Laemmlii buffer and analyzed. The samples were tested by PAGE, Commassie staining and Immunostaining.

$(NH_4)_2SO_4$ precipitation and GST purification (batch purification): The experiment was performed to examine if the purification of the sample using $(NH_4)_2SO_4$ purification would enhance the binding of the protein to the Glutathione column and thereby enhance the purification process.

A 3 ml sample of Takeout-GST fusion protein in Tni medium was precipitated using $(NH_4)_2SO_4$ and subjected to GST batch purification (BD BioSciences). The GST-purified samples were analyzed by Coomassie staining.

Optimizing $(NH_4)_2SO_4$ precipitation of Takeout-GST using different pH values: The $(NH_4)_2SO_4$ precipitation was carried out under different pH values to optimize binding to the GST column. The two pH values used were pH6.9 and pH 9.0. Takeout-GST fusion protein expressed in Tni medium was used for the experiment.

Two milliliters of protein sample was used per pH value. The protein was $(NH_4)_2SO_4$ precipitated and dialyzed against 1X PBS buffer of the respective pH value overnight. The protein samples

were purified through GST purification. The Glutatione beads were washed and equilibrated in 1X PBS buffer of the respective pH values. The fusion protein to Glutatatione Agarose bead binding step was extended to 3 hours and the first elution step extended for 1 hour. The remaining elution steps were maintained at 10 minutes each. The elution steps were pooled for each pH and stored at -20°C. The supernatants of the elution steps were also stored at -20°C.

The elution samples and supernatants for each pH value were concentrated and analyzed using Coomassie staining. The fusion protein elution samples and flow through samples from each pH were analyzed.

2.2.8. Processing of Takeout-GST fusion protein

The Takeout-GST fusion protein produced by Allele Biotechnology was cleaved using Thrombin. Digestion of the protein using Thrombin cleaves the GST tag off the fusion protein resulting in pure Takeout. The Takeout protein is then purified off the cleaved GST using Glutathione agarose.

62µg of protein (150 µl of protein sample) was dialyzed overnight into Thrombin cleavage buffer (50 mM Tris HCL pH=8, 150 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% β-mercaptoethanol).

The Thrombin powder (BD BioSciences. 12mg/ml. 1240U) was dissolved in Thrombin dilution buffer (BD BioSciences).

0.5 µl (0.62 U) of Thrombin solution was mixed well with the dialyzed protein. The reaction was kept O/N at room temperature.

The processed Takeout protein was purified using GST purification. The cleaved and removed GST protein and any un-cleaved protein are removed by attaching them to Glutathione agarose beads. The purified protein remains in the supernatant. GST and un-cleaved GST fusion protein was removed by directly adding 2 volumes of Glutathione coupled resin (BD BioSciences) to the reaction. The sample was incubated at 4°C for 30 minutes and centrifuged for 10 minutes at 5000 xg. The supernatant contained the purified cleaved takeout. The supernatant was stored at 4°C.

The digestion of the Takeout-GST fusion protein was analyzed using Coomassie staining, for quantification, and Immunostaining. The gel was stained using an affinity purified rabbit raised α-takeout antibody and visualized using a secondary HRP-conjugated α-rabbit antibody.

2.2.9. Equilibrium dialysis to detect binding of tritium labeled Juvenile hormone (JH^{H3}) to Takeout

We followed a protocol provided by Dr. W.G. Goodman (University of Wisconsin, Madison).

The processed and purified takeout protein was used for a protein ligand binding assay with JH^{H3} (PerkinElmer. NET15860500C. 50uC.18.5mBQ).

The amount of protein used in the assay- 64.8µg

The amount of JH^{H3}used in the assay- 1500 dpm

A 250 ml Erlenmeyer flask was coated with polyethelene glycol (20 MW). The radio-labeled Juvenile hormone was added to the flask and the solvent was evaporated using vacuum (Eppendorf. Vacufuge).

A volume of 100 ml of Tris buffer (50mM Tris, 100mM KCl, pH 6.8-7.2) was added to the vessel and swirled gently.

The 1 ml of Takeout protein (which had been dialyzed into binding buffer) was placed into a dialysis tubing (Snakeskin™ dialysis tubing. ThermoScientific) and clipped securely. The sample was then placed in the assay flask containing the JH^{H3} Juvenile hormone and binding buffer at 4°C for 4 days on a stirrer. Samples of 200µl each of the protein sample and binding buffer were analyzed for the presence of JH^{H3}, using a scintillation counter. A higher amount of dissociation counts at the protein sample compared to the buffer sample would indicate specific binding between the protein and JH^{H3} (subtracting the outside from the inside counts gives specific binding). The experiment was done twice.

CHAPTER III

RESULTS I

CHAPTER III: RESULTS I - JUVENILE HORMONE KNOCKDOWN AND COURTSHIP BEHAVIOR

3.1. Overview

Several factors are well established as requirements for normal male courtship behavior in *Drosophila melanogaster*. The master courtship regulators, Fru and Dsx are expressed in the nervous system, probably forming the courtship neural circuits (Kimura et al., 2005; Manoli et al., 2005; Stockinger et al., 2005; Yu et al. 2010). In addition, it is becoming increasingly clear that tissues outside the brain are also producing factors that are required for courtship (Bray and Amrein, 2003; Lazareva et al., 2007). One of them is the fat body that has been shown to produce male-specific factors required for courtship (Lazareva et al., 2007).The best studied among these factors is Takeout, a protein expressed in the head fat body of adult males. It is secreted into the hemolymph and plays a role in courtship as a secreted factor (Dauwalder et al., 2002; Lazareva et al., 2007). Since Takeout is secreted into the hemolymph, in order to affect courtship behavior, it needs to interact with the courtship neural circuits. The mechanisms of this interaction are unknown.

Analysis of the protein sequence of Takeout has shown that the protein is most similar to JHBPs of several insects (Dauwalder et al., 2002; Noriega et al., 2006; So et al., 2000). No JHBPs have been identified in *Drosophila* yet. This raises the possibility that Takeout might be acting as a JHBP in the hemolymph of the fly. If Takeout is acting as a JHBP, it can be hypothesized that that it acts as a carrier/ transporter for Juvenile hormone and transports it to the brain to interact

with the courtship neural circuits. If this hypothesis is valid, normal Juvenile hormone levels will be important for normal courtship behavior in flies.

To test this hypothesis, my project aimed to reduce Juvenile hormone levels in flies and see if this results in reduced courtship. To reduce Juvenile hormone levels in the fly, we targeted the enzyme JHAMT that acts in the hormone's biosynthesis pathway in converting Farnesoic acid to Methyl Farnesoate; the penultimate step in the pathway (Figure 2). The project aimed to reduce the levels of this enzyme by RNAi in order to disrupt synthesis of the hormone.

Two *GAL4* driver lines were used in the study: The *JHAMT-GAL4* line created for the study (as described above), and a universally expressed inducible *hsp70-GAL4* driver. The *hsp70-GAL*4 line was used for conditional knockdown of *JHAMT* in adult flies.

Two *JHAMT-RNAi* lines, which are dsRNAi vectors are available at the Vienna *Drosophila* RNAi Center (VDRC).

1. Transformant ID 19172; Inserted in the 2nd chromosome
2. Transformant ID 103958; Inserted in the 3rd chromosome

Preliminary experiments were performed on these RNAi lines to decide on one line to use for future experiments; *hsp70-GAL4* was used to drive expression of the two *UAS-JHAMT-RNAi* lines in two independent experiments. The resulting flies were subjected to a courtship assay. *JHAMT* RNAi performed using *UAS-JHAMT-RNAi* Transformant ID 19172 produced a higher reduction in courtship index. Therefore, it was used in further studies.

Several experiments were performed to reduce Juvenile hormone by *JHAMT-RNAi*:

1. Universal knockdown of Juvenile hormone levels in flies using the *JHAMT-GAL4* and *UAS-JHAMT-RNAi* at room temperature

2. Conditional Knockdown using the *Gal80^{ts}* system

3. Conditional Knockdown using the *hsp70-GAL4* driver

To verify that any observed effect is indeed due to a reduction in Juvenile hormone levels, rescue experiments were performed using the Juvenile hormone analog Methoprene.

Additionally, since reduction of Juvenile hormone by RNAi targeting of *JHAMT* was unlikely to be complete, we also performed experiments to conditionally ablate the Corpora allata in flies (the site of synthesis of Juvenile hormone). In these experiments, the *JHAMT-GAL4* driver was used to conditionally express the "cell death" gene construct, *UAS-HID*. It has been shown to cause apoptosis of the cells in which it is expressed (Gruntenko et al., 2010; Gruntenko et al., 2012). In addition *UAS-DTI* (diphtheria toxin) was used for ablation studies.

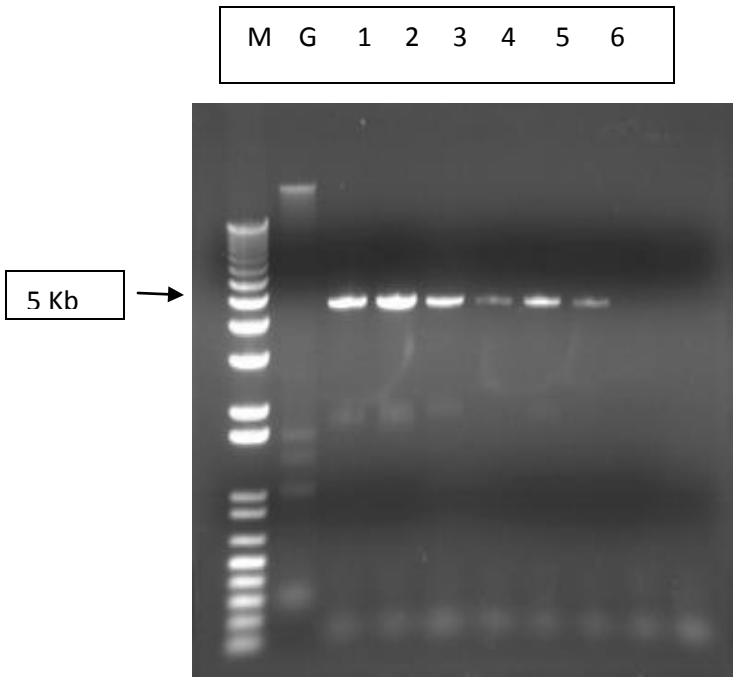
3.2. Generation of *JHAMT-GAL4* transgenic flies

Several *GAL4* lines have been described that are expressed in the *Drosophila* larval Corpora allata (Gruntenko et al., 2010; Gruntenko et al., 2012). However, we did not observe expression in adult Corpora allata when we tested them. Therefore, I created a transgenic fly line to

specifically target the Corpora allata to perform directed RNAi using a *UAS-JHAMT-RNAi* construct.

A 5043b *JHAMT* putative promoter region was amplified from genomic DNA extracted from wildtype CS flies. The fragment contains 5043b of upstream sequence up to the first AUG. The region was amplified using a two step PCR. The amplified region was cloned into the pSC-B vector (Stratagene®) and subsequently cloned into the pPTGAL *GAL4* transformation vector (Sharma et al., 2002). The sequence was verified by sequencing and the construct was injected into w^{1118} embryos to generate transgenic flies.

(A)



(B)

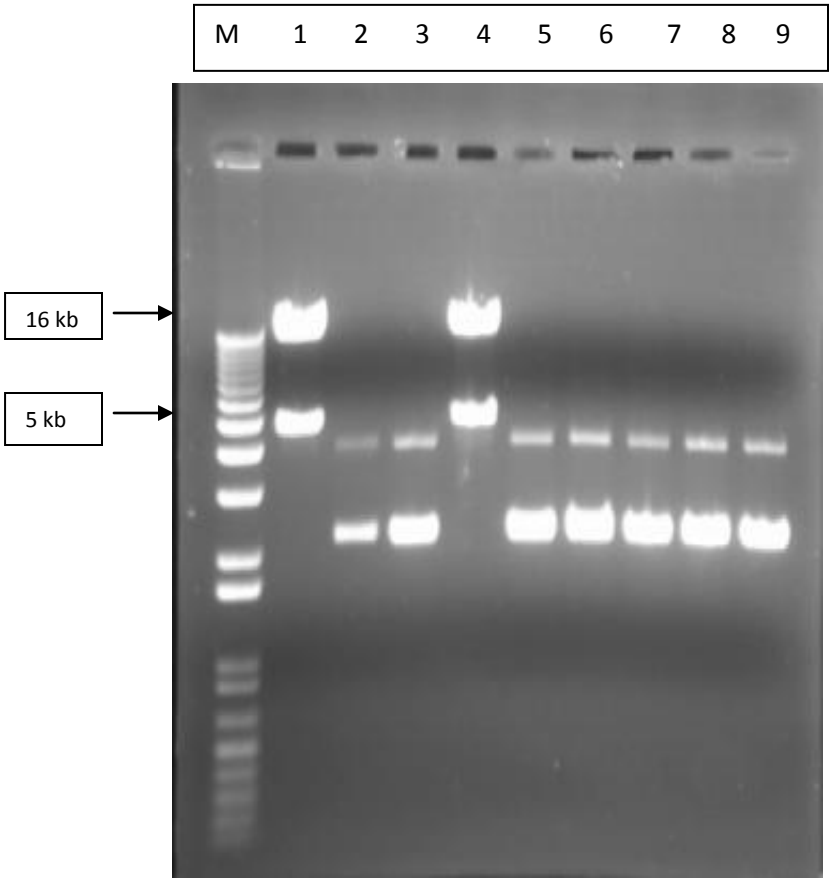


Figure 17: Cloning of the *JHAMT* upstream promoter region into the pPTGAL vector. (A) Amplification of the *JHAMT* upstream promoter region using two step polymerase chain reaction. M= Marker; G= Genomic DNA; 1-6- PCR reaction samples. Arrow indicates band of 5.0 Kb length. (B) The cloning of the 5043b *JHAMT* promoter region into the 11 Kb pPTGAL vector results in a 16.2 Kb construct [Appendix 1 E]. The cloning was verified by subjecting the construct to a restriction test digest, using Not1 and BamH1. Several mini-preps were prepared and the colonies subjected to a restriction test digest. This resulted in the release of the 5.2 Kb insert from the pPTGAL vector. Samples 1 and 4 of minipreps indicate the presence of the construct.

The 5043 b *JHAMT* upstream promoter region was cloned into the 4.3 kb pSC-B vector. The pSC-B + *JHAMT* promoter construct was digested using Not1 and BamH1 to release the insert from the vector. The 5043 b insert band was excised and removed from the gel for ligation into Not1/BamH1 digested pPTGAL4 (Figure 17).

DNA was prepared using the QIAGEN midi prep protocol and the sequence of the insert was verified by sequencing. The construct was then injected into w^{1118} fly embryos to create transgenic flies.

3.2.1. Mapping the fly lines

The 14 fly lines obtained were mapped to locate the chromosomes containing the construct.

Fly line	Chromosome	Comments
1	X chromosome	homozygous
2	2nd chromosome	homozygous
3	X chromosome	homozygous
4	Lethal	
5	2nd chromosome	balanced
6	3rd chromosome	homozygous
7	3rd chromosome	homozygous
8	Lethal	
9	2nd chromosome	balanced
10	3rd chromosome	Homozygous
11	2nd chromosome	balanced
12	X chromosome	homozygous
13	Lethal	
14	2nd chromosome	balanced

Table 2- The chromosomes containing the *JHAMT-GAL4* construct. The transgenic fly lines containing the construct were mapped to locate the chromosomes harboring the insert.

The lines indicated were made homozygous. The others were homozygous lethal and were kept with a balancer chromosome.

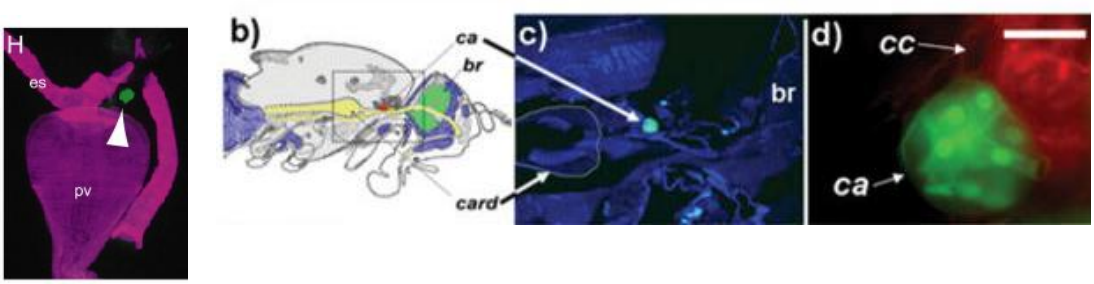
3.2.2. Screening of fly lines- X-GAL staining

In order to verify expression of the *JHAMT-GAL4* transgene, each of the fly lines was used to drive expression of a *LacZ* gene. The GAL4- UAS system was used, where the *JHAMT-GAL4* driver was expected to drive the expression of the *UAS-LacZ* gene in the Corpora allata. Fly lines in which the *JHAMT-GAL4* driver expression is strong and specific were selected. The selected lines are candidate lines for further behavior experiments.

Each of the fly lines was crossed to the *UAS-LacZ III* fly line to create flies containing the *JHAMT-GAL4* driver and the *UAS-LacZ* construct. The expression of the *LacZ* gene results in synthesis of β-galactosidase (β-gal). The flies were freeze-sectioned, fixed, and stained with X-GAL. X-GAL is a substrate for β-gal and marks the *GAL4*-expressing cells with a blue stain. The tissue specificity and strength of expression of the *JHAMT-GAL4* driver was examined for each transgenic fly line. The results for each transgenic line are shown in Figure 18.

The transgenic fly line 6 was chosen for its strong expressing of β-gal in the region of the Corpora allata of the adult fly with minimum background staining.

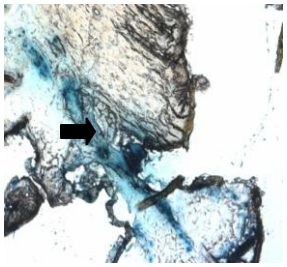
(A)



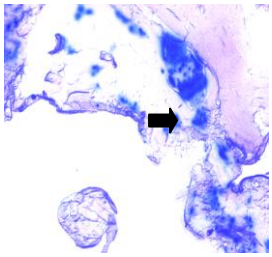
Niwa et al., 2008

Belgacem and Martin, 2007

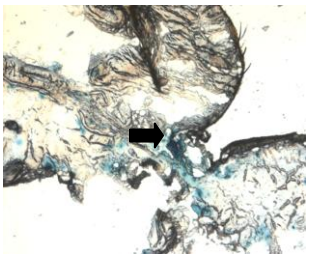
(B)



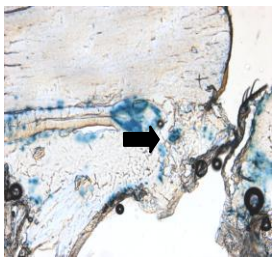
Line 2



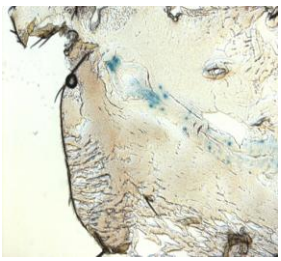
Line 3



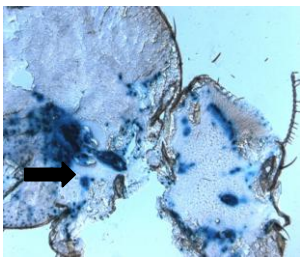
Line 5



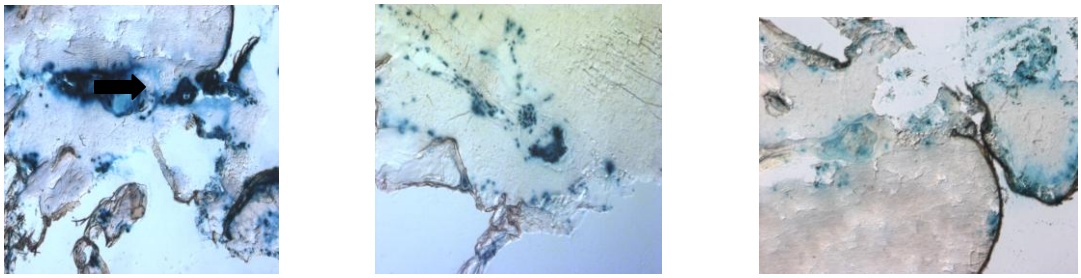
Line 6



Line 7



Line 9



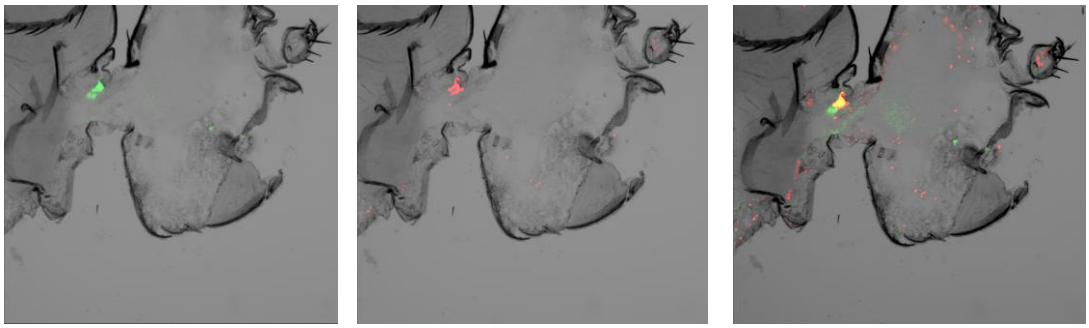
Line 11 Line 12 Line 14

Figure 18: Screening of transgenic fly lines containing the *JHAMT-GAL4* construct. (A) The position of the Corpora allata in the adult *Drosophila* (Belgacem and Martin, 2007; Niwa et al., 2008). (B**)** X-GAL staining of sectioned transgenic fly lines containing the *JHAMT-GAL4* and *UAS-lacZ* construct. Each of the fly lines containing the *JHAMT-GAL4* transgene was crossed to the *UAS-lacZ* fly line. The male progeny were sectioned, stained with X-GAL and examined for the location of X-GAL staining, indicating the location of *JHAMT-GAL4* expression. (Arrows indicate position of the Corpora allata). Based on the screening, fly line 6 (indicating specific staining in the Corpora allata, and minimal background staining) was selected for further studies.

3.2.3. Verification of *JHAMT-GAL4* expression in the Corpora allata using immunohistochemistry

To further verify the expression of the *JHAMT-GAL4* driver in the Corpus allatum of the selected fly line, Line 6, the flies carrying the driver and *UAS-LacZ* construct were sectioned and subjected to immunohistochemistry. Previous research had used a α-JHAMT antibody to visualize the Corpora allata in the adult fly (Niwa et al., 2008). Male flies carrying the two constructs were freeze-sectioned, fixed and stained using antibodies against JHAMT (α-JHAMT) and β-Gal (α-β-Gal). Sections were observed for overlap in expression of β-Gal and JHAMT proteins in the Corpora allata. We observed overlapping staining, indicating the expression of *JHAMT-GAL4* in the Corpora allata (Figure 19).

(A)



(B)

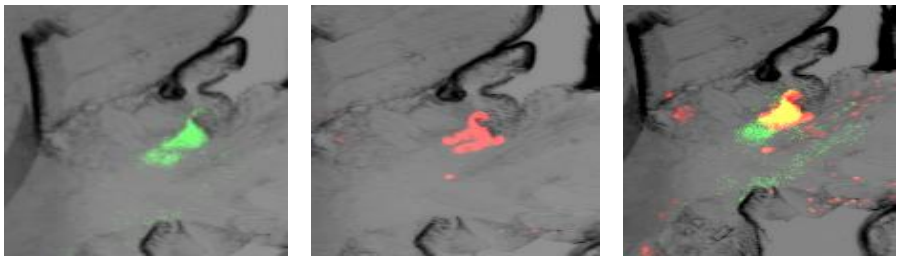


Figure 19: Immunohistochemistry of fly sections of line 6 using α-JHAMT and α-β-Gal. (A) 10X magnification. (B) 60X magnification. Male flies of fly line 6 were sectioned to 20µm and stained against the two proteins JHAMT and β-Gal. The overlap in expression confirms the expression of the *JHAMT-GAL4* driver in the Corpora allata. α-JHAMT (green); α- β-gal (red); overlay (yellow).

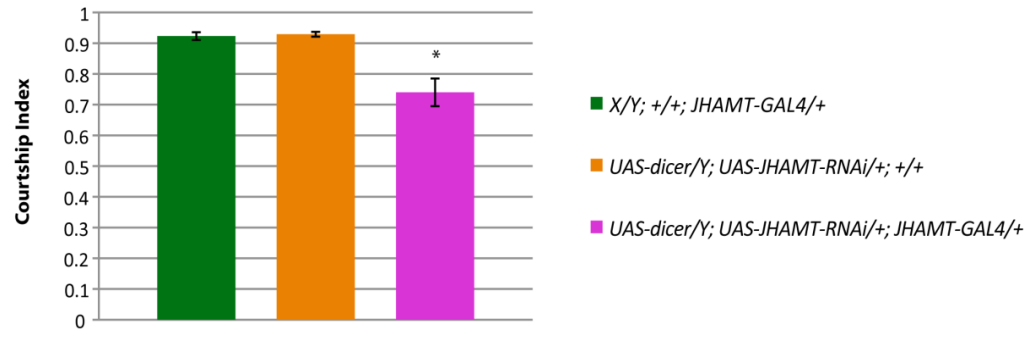
3.3. Wildtype Juvenile hormone levels are required for normal male courtship

3.3.1. RNAi-mediated disruption of the Juvenile hormone synthesis pathway results in a courtship mutant phenotype

The Corpora allata specific *JHAMT-GAL4* driver was used to reduce JHAMT levels in flies by expressing *UAS-JHAMT-RNAi*. The experimental genotype contained the driver and the *UAS-JHAMT-RNAi* constructs. To increase the effect of RNAi, a *UAS-dicer* construct was included in the genotypes (Dietzl et al., 2007). The experimental genotype was *UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+* containing all three constructs. The control genotypes were *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+* and *X/Y; +/+; JHAMT-GAL4/+*. Courtship assays were performed as described in section 2.1.10 (Materials and Methods). The experiment was performed to 10 replicates.

The results are shown in Figure 20. The experimental genotype, wherein the disruption of Juvenile hormone synthesis is expected, showed an approximate reduction of 0.3 in courtship index. This reduction in courtship is significant ($p < 0.001$, ANOVA) in comparison to the control genotypes. The control genotypes have the *JHAMT-GAL4* and *UAS-JHAMT-RNAi* constructs in two separate fly lines. The control genotypes had a >0.9 courtship index. The mutant males showed a reduced ability to sustain the courtship behavior towards the females, showing short periods of distraction within the observation period. The mutant males were able to perform all of the courtship steps, but performed them less often, when compared to the control males (data not shown).

(A)



(B)

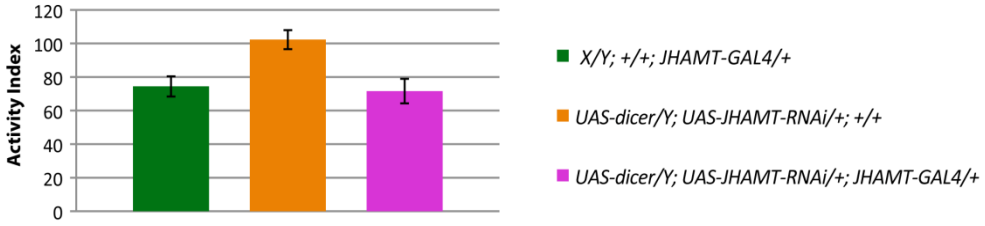


Figure 20: Expression of *JHAMT-RNAi* in the Corpora allata reduces male courtship behavior. (A) Courtship index of experimental (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*) and controls (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+* and *X/Y; +/+; JHAMT-GAL4/+*) genotypes towards wildtype CS females. The experimental genotype shows a significant reduction in courtship index as indicated by asterix* ($p < 0.001$). (B) Activity assay of the experimental and control genotypes. The activity of the mutant flies is comparable to that of controls indicating that the reduced courtship is not a result of a locomotor defect (n=10. Error bars represent s.e.m).

Flies from the experimental and control genotypes were also subjected to an Activity/ Locomotor assay, to verify that the reduced courtship index is not due to a reduced locomotor ability.

One of the controls (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+*) has an activity index that is statistically higher than the other control (*X/Y; +/+; JHAMT-GAL4/+*) (p=0.0045) and the experimental genotype (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*) (p=0.0021). However, the courtship index of the experimental genotype (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*) is significantly lower than that of the control with a similar activity index, making it unlikely that the courtship defect is caused by general sickness of the experimental genotypes.

These results suggest that the disruption of Juvenile hormone synthesis reduces the courtship index/ courtship behavior of *Drosophila melanogaster* males. They also suggest that the reduction is not a result of reduced locomotor activity.

3.3.2. Methoprene treatment of Juvenile hormone synthesis disrupted flies results in rescue of the mutant phenotype

To confirm that the *JHAMT-RNAi* expression in the above knockdown flies results in a reduction of Juvenile hormone levels, and that this is the cause for the reduction in courtship index of the flies, the mutant and control flies were treated with the Juvenile hormone analog, Methoprene. Methoprene has been used in several previous studies as a substitute for Juvenile hormone (Dubrovsky et al., 2002; Liu et al., 2009; Wilson et al., 2006). Methoprene treatment was

performed on the experimental (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*) and control (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+* and *X/; +/+; JHAMT-GAL4/+*) genotypes. The flies were created and raised as in the above experiment. They were matured for 4 days and treated with 1ul of 0.5 mg/µl Methoprene dissolved in 100% Acetone (which is a common diluent for Methoprene). The flies were then kept at room temperature for 4 hours and then tested in a courtship assay. As a treatment control, the flies of the three genotypes were treated with pure Acetone. This would indicate if Acetone has an effect on the behavior on its own. The treated and un-treated flies (flies treated with 100% Acetone only) of the all genotypes were subjected to a courtship assay. The results are shown in Figure 21.

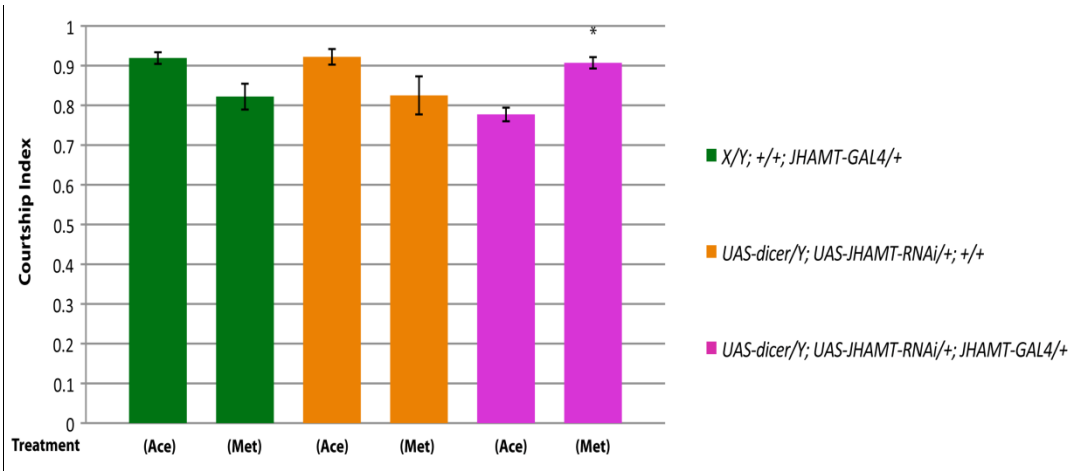


Figure 21: Methoprene treatment of Juvenile hormone synthesis disrupted flies rescues the courtship mutant phenotype. The experimental genotype (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*) and control (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+* and *X/Y; +/+; JHAMT-GAL4/+*) genotypes were Methoprene treated. The experimental genotype shows a significant increase in courtship index by Methoprene treatment compared to Acetone treatment as indicated by asterix* (p= 0.001) (n=20. Error bars represent s.e.m). (Treatment; Acetone= (Ace), Methoprene= (Met)).

The results show that the treatment of the mutant flies with Methoprene results in an increase in their courtship index to that of the control flies (Figure 21). This suggests that the RNAi machinery targeting *JHAMT* causes the disruption of Juvenile hormone synthesis, leading to reduced levels of Juvenile hormone. When the Juvenile hormone analog Methoprene is applied to the flies, the courtship reduction is rescued back to normal.

The treatment of the control genotypes with Acetone does not have an effect on courtship phenotype (Figure 21). Both control genotypes indicate a normal above 0.9 courtship index, upon Acetone treatment. Additionally, the experimental *JHAMT* knockdown genotype indicates a 0.7 courtship index when treated with Acetone. Collectively, these observations indicate that Acetone per se does not have a courtship effect.

Another observation of the experiment is the reduced level of courtship seen when the control genotypes are treated with Methoprene (Figure 21). This suggests that under normal levels of Juvenile hormone, additional Juvenile hormone or Juvenile hormone analog, Methoprene has the effect of leading to a reduced courtship phenotype. However, this phenotype failed to indicate statistical significance.

3.3.3. Conditional induction of *JHAMT* RNAi by the *Gal80^{ts}* system

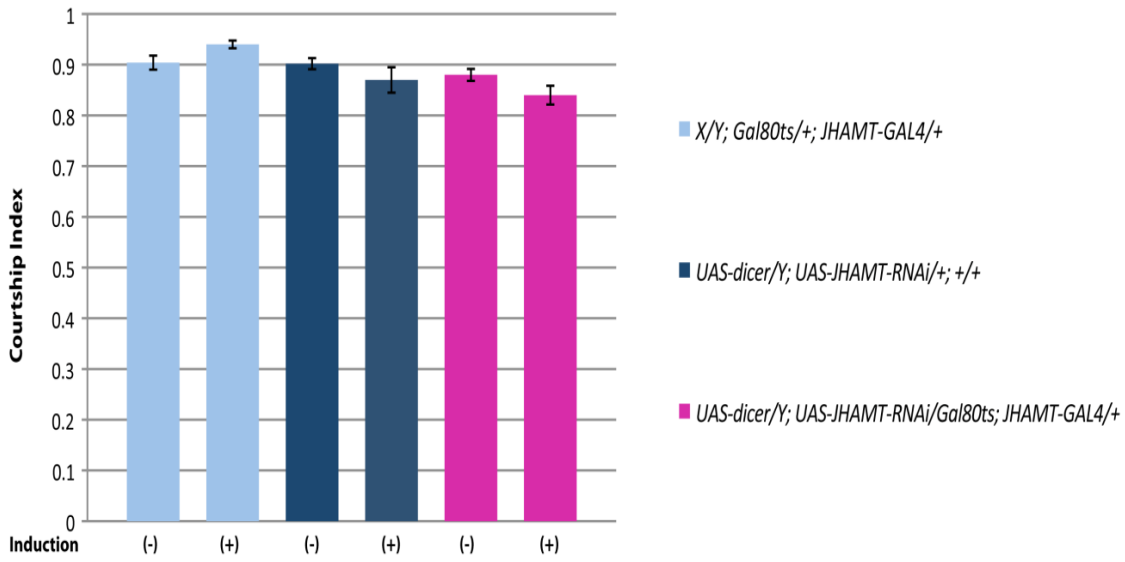
Juvenile hormone is a major hormone acting in the development and life cycle of an insect. The hormone primarily functions in maintaining the larval stages between molts/ stages of the life cycle, until the stage has reached optimal growth, development and critical weight characteristics of the stage. The disruption of this process is lethal. The fact that our *JHAMT*-

GAL4/UAS-JHAMT-RNAi flies survived to normal adulthood clearly indicates that the RNAi knockdown is weak; otherwise we would not have recovered adult males. Nevertheless, since our disruption of Juvenile hormone synthesis was throughout the life cycle of the fly, this could lead to developmental effects that might affect adult behavior.

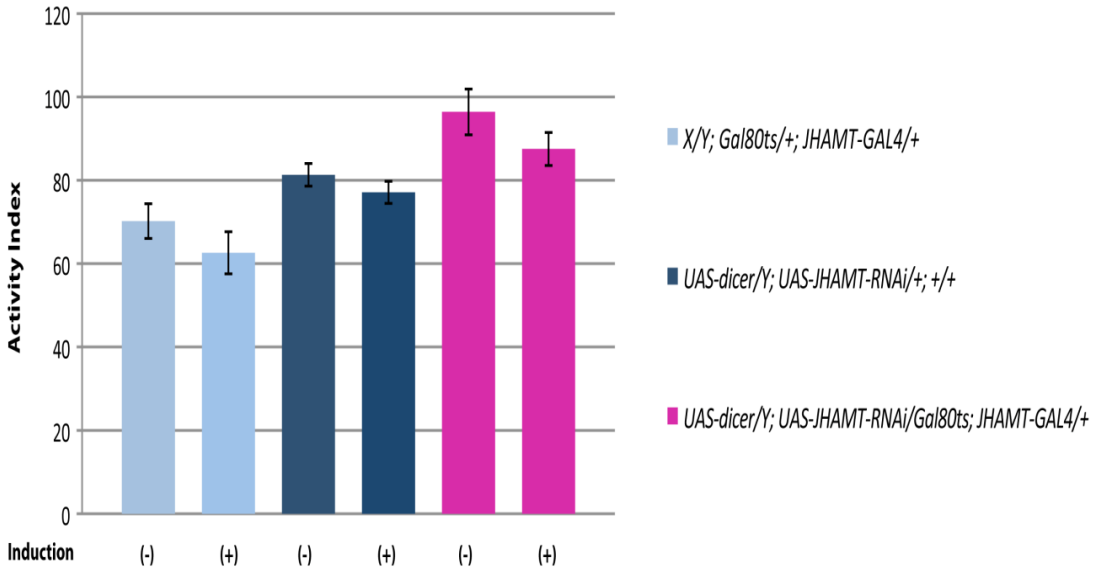
To address this issue, the inducible *Gal80^{ts}* system was used to reduce Juvenile hormone levels in flies after the development period, i.e. in the adult fly. Therefore, the experimental flies contained the *tubulin-Gal80^{ts}* transgene in addition to the *JHAMT-GAL4*, *UAS-JHAMT-RNAi* and *UAS-dicer* constructs. The experimental genotype was *UAS-dicer/Y; UAS-JHAMT-RNAi/ Gal80^{ts}; JHAMT-GAL4/+*. The control genotypes used were *X/Y; Gal80^{ts}/+; JHAMT-GAL4/+* and *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+*.

Gal80^{ts} expression is driven by the universally expressed Tubulin promoter. *Gal80^{ts}* is active at 18°C to prevent the activity of *GAL4* on the *UAS-JHAMT-RNAi* and *UAS-dicer* constructs. Therefore, at the restrictive temperature for RNAi expression, 18°C, it is not expressed. The activity of *Gal80^{ts}* is prevented at the permissive temperature, 32°C, and *GAL4* is active to drive the expression of the UAS constructs. This system permits normal development of the flies at 18°C. Eclosed adult flies were maintained at 18°C for 7 days for sexual maturation and then transferred to 32°C for two days to induce the RNAi against *JHAMT*. The flies were then assayed for their courtship behavior and locomotor activity.

(A)


Courtship Index
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Induction
(-)
(+)
(-)
(+)
(-)
(+)
X/Y; Gal80ts/+; JHAMT-GAL4/+
UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+
UAS-dicer/Y; UAS-JHAMT-RNAi/Gal80ts; JHAMT-GAL4/+

(B)


Activity Index
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100
80
60
40
20
0
Induction
(-)
(+)
(-)
(+)
(-)
(+)
X/Y; Gal80ts/+; JHAMT-GAL4/+
UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+
UAS-dicer/Y; UAS-JHAMT-RNAi/Gal80ts; JHAMT-GAL4/+

Figure 22: Conditional disruption of Juvenile hormone synthesis using *Gal80^{ts}*. (A) Courtship index of induced and un-induced experimental (*UAS-dicer/Y; UAS-JHAMT-RNAi/ Gal80^{ts}; JHAMT-GAL4/+*) and control (*X/Y; Gal80^{ts}/ +; JHAMT-GAL4/+* and *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+*) genotypes towards CS females. The induced experimental genotype does not show a significant reduction in courtship index. (B) Activity assay of the experimental and control genotypes. The activity of the mutant flies (induced experimental genotype) is not deficient compared to that of controls (n=10. Error bars represent s.e.m). (Induction; Un-induced= (-), Induced= (+)).

The experimental genotype where *JHAMT* RNA knockdown is expected contained the *UAS-JHAMT-RNAi*, *UAS-dicer* and *JHAMT-GAL4* constructs along with the $Gal80^{ts}$ to conditionally express the RNAi machinery in adults. The controls contained the *UAS* constructs and *JHAMT-GAL4* construct in two different lines. The courtship assay does not indicate a statistically significant reduction in courtship index. The reduction of courtship in the experimental genotype was not as strong as that observed when the Juvenile hormone synthesis was reduced throughout development (Figure 22). This indicates that the conditional induction regimen was not as strong and the level of knockdown may not be sufficient to reduce the courtship index, or that adult disruption of *JHAMT* has no effect on courtship.

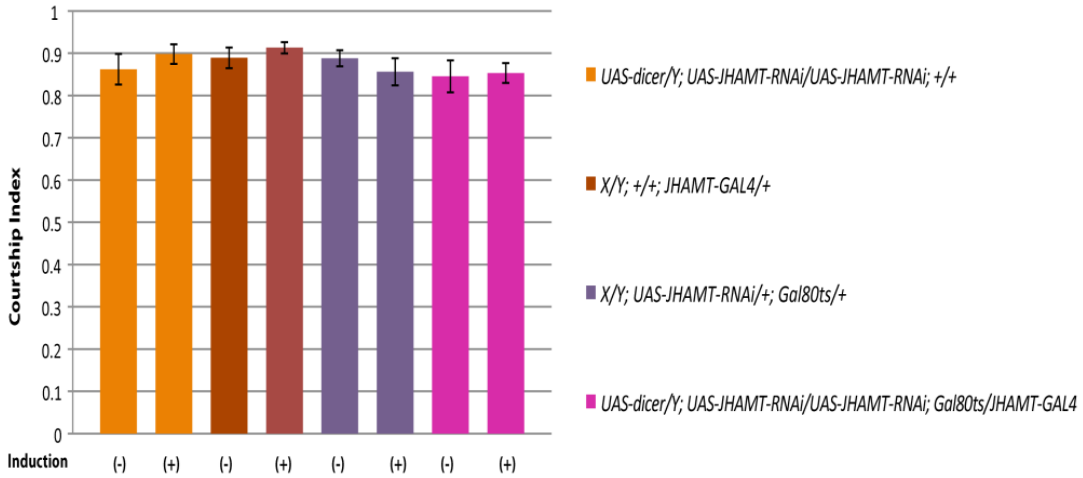
Also, one of the control genotypes, *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+* shows a reduced courtship index after induction as compared un-induced males. This is likely due to genetic background issues. Alternatively, the *UAS-JHAMT-RNAi* construct itself could have some leaky expression.

To increase the level of conditional knockdown of Juvenile hormone synthesis in adult flies, knockdown was performed with genotypes containing two copies of *UAS-JHAMT-RNAi*. A new experiment was carried out using the experimental *UAS-dicer/Y; UAS-JHAMT-RNAi/UAS-JHAMT-RNAi; JHAMT-GAL4/*$Gal80^{ts}$. The control genotypes were, *UAS-dicer/Y; UAS-JHAMT-RNAi/ UAS-JHAMT-RNAi; +/+* and *X/Y; +/+; JHAMT-GAL4/+* and *X/Y; UAS-JHAMT-RNAi/+;* $Gal80^{ts}$*/+*.

The flies were raised and matured for 7 days at 18°C and induced for 2 days at 32°C. They were then rested at room temperature for one hour and subjected to a courtship assay. The experiment was done to 10 replicates. The courtship indices were analyzed using ANOVA. The

experimental and control flies were also subjected to a 3 minute activity assay to determine if any reduction in courtship levels could be due to a defect in locomotor activity.

(A)


Courtship Index
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Induction
(-) (+) (-) (+) (-) (+) (-) (+)
UAS-dicer/Y; UAS-JHAMT-RNAi/UAS-JHAMT-RNAi; +/+
X/Y; +/+; JHAMT-GAL4/+
X/Y; UAS-JHAMT-RNAi/+; Gal80ts/+
UAS-dicer/Y; UAS-JHAMT-RNAi/UAS-JHAMT-RNAi; Gal80ts/JHAMT-GAL4

(B)

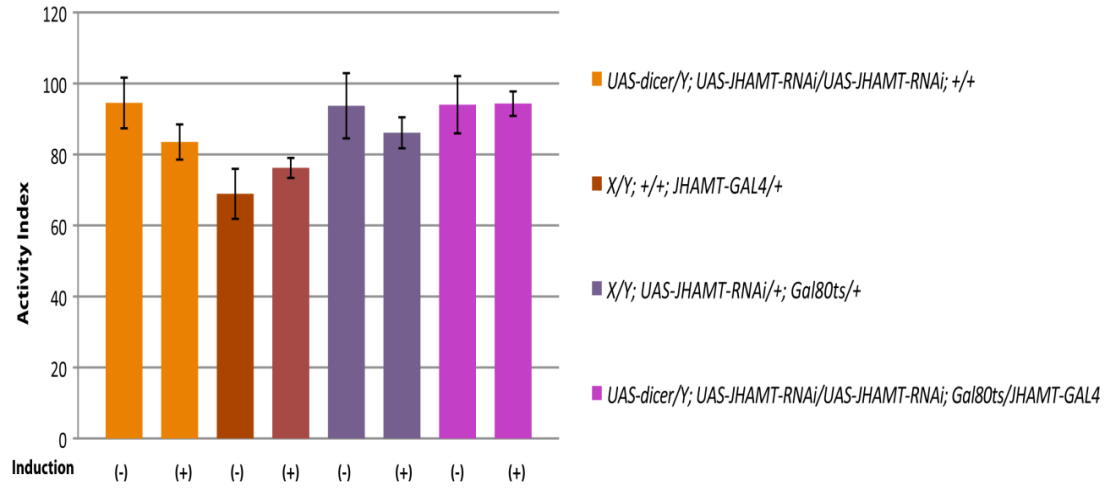

Activity Index
120
100
80
60
40
20
0
Induction
(-) (+) (-) (+) (-) (+) (-) (+)
UAS-dicer/Y; UAS-JHAMT-RNAi/UAS-JHAMT-RNAi; +/+
X/Y; +/+; JHAMT-GAL4/+
X/Y; UAS-JHAMT-RNAi/+; Gal80ts/+
UAS-dicer/Y; UAS-JHAMT-RNAi/UAS-JHAMT-RNAi; Gal80ts/JHAMT-GAL4

Figure 23: Conditional knockdown of Juvenile hormone synthesis in adult flies using two *Gal80^{ts}* constructs. (A) Courtship assay of experimental and control genotypes. The experimental genotype was *UAS-dicer/Y; UAS-JHAMT-RNAi/UAS-JHAMT-RNAi*; *JHAMT-GAL4/Gal80^{ts}*. The control genotypes were *UAS-dicer/Y; UAS-JHAMT-RNAi/ UAS-JHAMT-RNAi; +/+* and *X/Y; +/+; JHAMT-GAL4/+* and *X/Y; UAS-JHAMT-RNAi/+; Gal80^{ts}/+*. The induced experimental genotype does not show a significant reduction in courtship index. (B) Activity assay of the above flies do not show a defect in locomotion in induced experimental flies (n=10. Error bars represent s.e.m.). (Induction; Un-induced= (-), Induced= (+)).

We conclude from these experiments that the addition of a second copy of *UAS-JHAMT-RNAi* does not reduce the Juvenile hormone synthesis to a significant level in the conditional *Gal80^{ts}* induction system (Figure 23). As discussed before, the *JHAMT RNAi* is a weak RNAi. *Gal80^{ts} /Gal4* mediated induction is often not as efficient as just *GAL4* alone. We assume that taken together, Juvenile hormone levels were not lowered to the degree that has led to courtship defect in the un-conditional experiments.

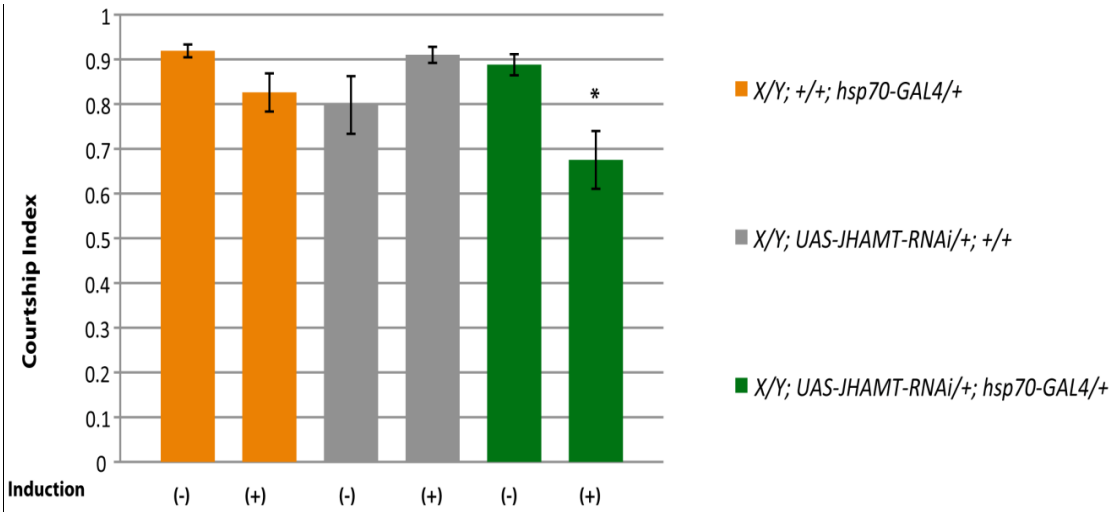
3.3.4. Conditional disruption of Juvenile hormone synthesis by heat-inducible *GAL4*-driven RNAi results in reduced courtship index

Conditional disruption of Juvenile hormone synthesis in adult flies using the *Gal80^{ts}* system did not result in the courtship reduction seen in unconditional *JHAMT-GAL4 / UAS-JHAMT RNAi* flies. As mentioned above, this might be because of less efficient expression of *JHAMT-RNAi* by the *GAL4* / *Gal80^{ts}* system. To further examine an adult requirement for Juvenile hormone in courtship, another inducible system was used. The *hsp70-GAL4* transgene expresses *GAL4* under the control of the heat-inducible promoter of *hsp70*. *hsp70-GAL4* is activated at 37°C, the induction temperature. *hsp70-GAL4* was used to conditionally express *UAS-JHAMT-RNAi* in adult flies.

The experimental genotype used was *X/Y; UAS-JHAMT-RNAi/+; hsp70-GAL4/+*, having the *hsp70-GAL4* driver to drive the expression of *UAS-JHAMT-RNAi*. The control genotypes contained one construct each, to indicate if they show a courtship phenotype on their own. The controls were *X/Y; +/+; hsp70-GAL4/+* and *X/Y; UAS-JHAMT-RNAi/+; +/+*. The flies were raised at 25°C and matured at the same temperature for 4 days. They were then induced for 1 hour at 37°C and

kept at room temperature for 4 hours. The flies, induced and un-induced experimental and control genotypes were subsequently subjected to a courtship assay. The experiment was done to 20 replicates. Furthermore, the above genotypes, induced and un-induced were subjected to a 3-minute activity assay to observe for locomotor defects.

(A)


Courtship Index
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Induction
(-)
(+)
(-)
(+)
(-)
(+)
X/Y; +/+; hsp70-GAL4/+
X/Y; UAS-JHAMT-RNAi/+; +/+
X/Y; UAS-JHAMT-RNAi/+; hsp70-GAL4/+

(B)

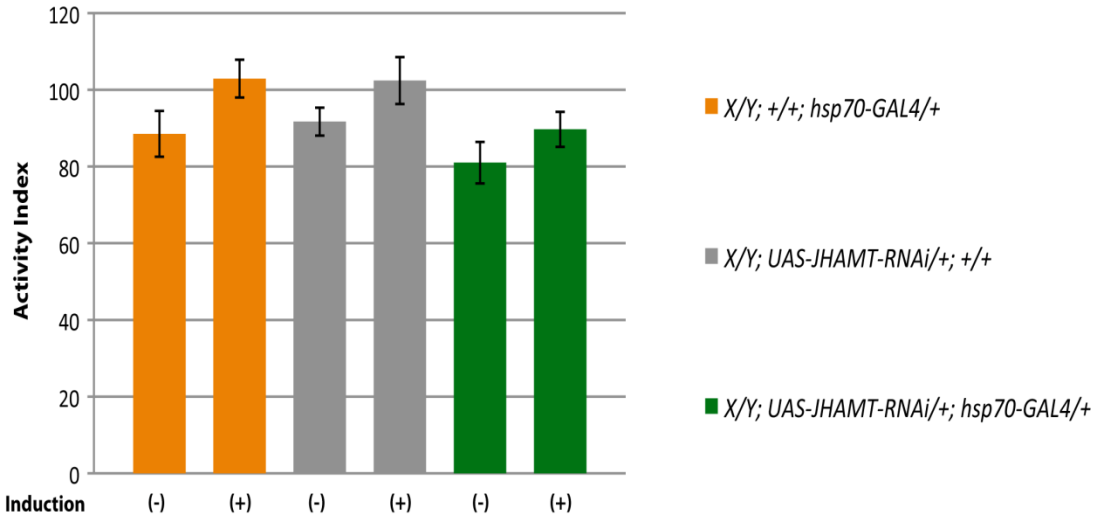

Activity Index
120
100
80
60
40
20
0
Induction
(-)
(+)
(-)
(+)
(-)
(+)
X/Y; +/+; hsp70-GAL4/+
X/Y; UAS-JHAMT-RNAi/+; +/+
X/Y; UAS-JHAMT-RNAi/+; hsp70-GAL4/+

Figure 24: Conditional disruption of Juvenile hormone synthesis using *hsp70-GAL4*-driven RNAi results in a reduced courtship index. (A) Courtship index of induced and un-induced experimental (*X/Y; hsp70-GAL4/ UAS-JHAMT-RNAi; +/+*) and control (*X/Y; hsp70-GAL4/+; +/+* and *X/Y; UAS-JHAMT-RNAi/+; +/+*) genotypes. The experimental genotype shows a significant reduction in courtship index as indicated by asterix* (p=0.0007. ANOVA) (n=20. Error bars represent s.e.m.) Induced flies were heat-shocked at 37^{o}C for 1 hour and let recover for four hours. (B) Activity assay of the induced and un-induced genotypes (n=10. Error bars represent s.e.m.). (Induction; Un-induced= (-), Induced= (+)).

The courtship assay indicates a significant (p=0.0007) reduction in courtship index in the induced experimental flies when compared to the un-induced experimental and induced and un-induced control genotypes (Figure 24). The reduction in courtship index in the induced experimental genotypes *hsp70-GAL4 / UAS-JHAMT-RNAi* was slightly more than that observed for the courtship reduction obtained in *JHAMT-GAL4 / UAS-JHAMT-RNAi*. This is likely due to the *hsp70-GAL4* driver being a stronger driver than *JHAMT-GAL4*. The un-induced X*/Y; UAS-JHAMT-RNAi/+; +/+* control and the induced *X/Y; +/+; hsp70-GAL4/+* control genotypes have given relatively low courtship indices when compared to the remaining control genotypes. However, they do not show a statistical significance when compared to their induced and un-induced counterparts respectively; *X/Y; UAS-JHAMT-RNAi/+; +/+* (UI) vs *X/Y; UAS-JHAMT-RNAi/+; +/+* (I) and *X/Y; hsp70-GAL4; +/+* (UI) vs *X/Y; +/+; hsp70-GAL4/+* (I).

The activity assay for the experiment indicates a comparable level of activity between the induced experimental and the un-induced states of the control genotypes. This indicates that the reduction in courtship index of the induced experimental is not a result of a locomotor defect.

3.3.5. Methoprene treatment of *hsp70-GAL4*-driven *JHAMT* RNAi flies results in rescue of the mutant phenotype

To confirm that the conditional courtship mutation caused by heat-shocked *GAL4* (*hsp70-GAL4*)-induced RNA interference against *JHAMT* is the result of a reduction of Juvenile hormone levels, the heat-induced flies were Methoprene treated. The experimental (*X/Y; UAS-JHAMT-RNAi/+; hsp70-GAL4/+*) and control genotypes (*X/Y; +/+; hsp70-GAL4/+* and *X/Y; UAS-JHAMT-RNAi/+;*

+/+) genotypes were heat induced at 37°C for 1 hour and kept at room temperature to rest for 1 hour. The flies of experimental and control genotypes were treated with Methoprene. As a control, the induced flies not treated with Methoprene were treated with 100% Acetone. The flies were then kept at room temperature for 4 hours and subjected to courtship assay. The experiment was done to 20 replicates.

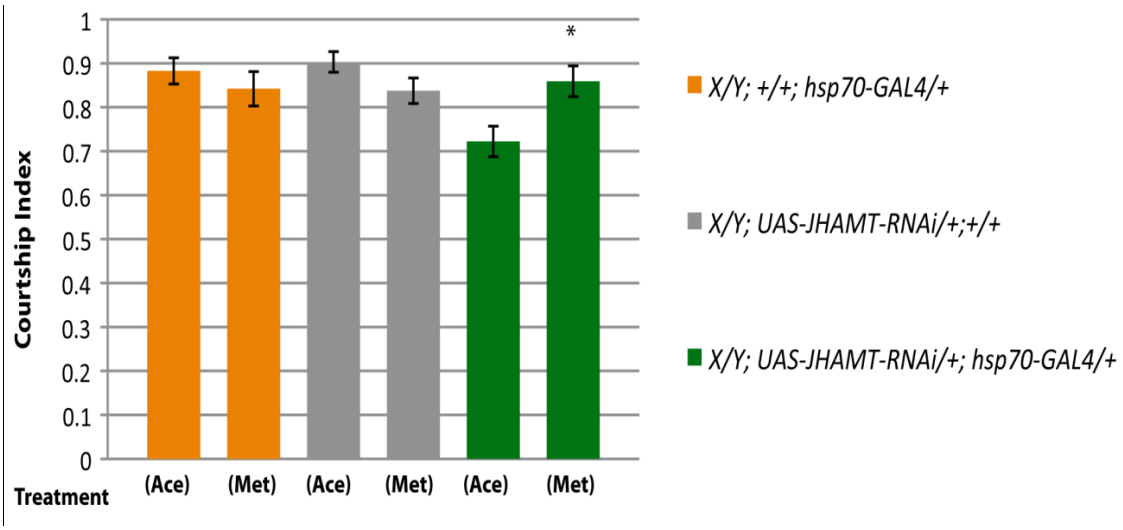


Figure 25: Methoprene treatment of Juvenile hormone synthesis disrupted flies results in rescue of the courtship mutant phenotype. The experimental genotype (*X/Y; UAS-JHAMT-RNAi/+; hsp70-GAL4/+*) and control (*X/Y; +/+; hsp70-GAL4/+* and *X/Y; UAS-JHAMT-RNAi/+; +/+*) genotypes were induced at 37°C and Methoprene treated. The experimental genotype shows a significant increase in courtship index as indicated by asterix* ($p < 0.003$) (n=20. Error bars represent s.e.m). (Treatment; Acetone= (Ace), Methoprene= (Met)).

Methoprene treatment of the flies where RNAi against *JHAMT* was carried out conditionally using *hsp70-GAL4* indicates a rescue of the courtship mutant phenotype to that of the control (Acetone-treated) genotypes (Figure 25). This suggests that the RNAi machinery targeting *JHAMT* causes the disruption of Juvenile hormone synthesis, conditionally in adult flies, leading to reduced levels of Juvenile hormone. When the Juvenile hormone analog Methoprene is applied to the flies, the courtship reduction is rescued back to normal.

As observed in the previous rescue experiment (Figure 21), while the courtship index of the experimental flies is rescued by Methoprene to that of the control genotypes, the treatment of the control genotypes with Methoprene appears to decrease the courtship index, indicating an effect leading to a reduced courtship phenotype.

3.3.6. *JHAMT-GAL4*-driven *JHAMT* RNAi and a *takeout* heterozygous mutant state does not show genetic interaction

Previous research has indicated a role for *takeout* and the fat body, in courtship behavior in *Drosophila melanogaster* (Dauwalder et al., 2002; Lazareva et al., 2007). The current experiments suggest that optimal Juvenile hormone levels are essential for normal courtship behavior. Since *takeout* is most similar to JHBPs (Dauwalder et al., 2002; Noriega et al., 2006; So et al., 2000), we next wanted to explore the possibility of a genetic interaction between *takeout* and Juvenile hormone levels in courtship. To explore this possibility, three experimental genotypes were compared in this experiment.

1. to^1 homozygous mutants (*UAS-dicer/Y; UAS-JHAMT-RNAi/+;* to^1/to^1)

2. Juvenile hormone knockdown mutants (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*)
3. Juvenile hormone knockdown mutants in a heterozygous to^1 background (*UAS-dicer/Y; UAS-JHAMT-RNAi/+;* $to^{1/}$*JHAMT-GAL4*)

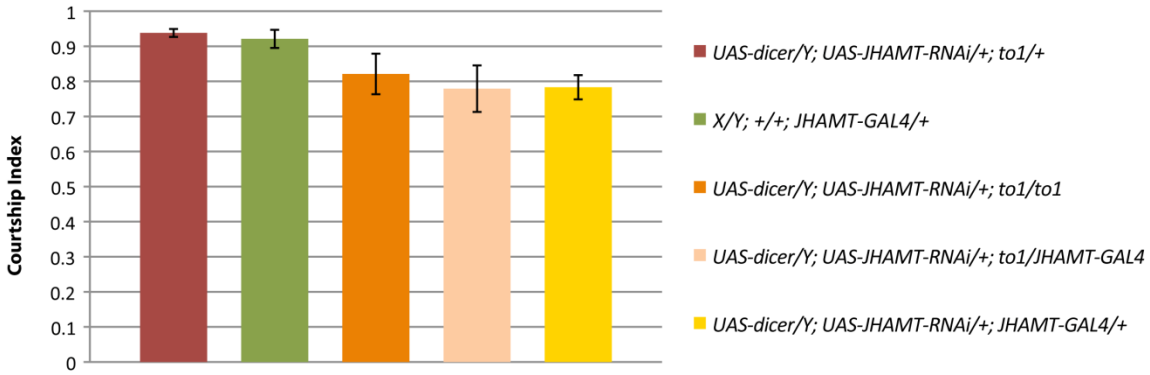
Two control genotypes were used:

1. *X/Y; +/+; JHAMT-GAL4/+*
2. *UAS-dicer/Y; UAS-JHAMT-RNAi/+;* to^1*/+*

The control genotypes were arranged to keep the *UAS* and *JHAMT-GAL4* constructs in two separate fly lines and to confirm previous data showing that to^1 heterozygous shows a normal courtship phenotype.

The flies were created and raised at room temperature and selected virgin males were matured for 4 days. They were then subjected to a courtship assay.

(A)


Courtship Index
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
UAS-dicer/Y; UAS-JHAMT-RNAi/+; to1/+
X/Y; +/+; JHAMT-GAL4/+
UAS-dicer/Y; UAS-JHAMT-RNAi/+; to1/to1
UAS-dicer/Y; UAS-JHAMT-RNAi/+; to1/JHAMT-GAL4
UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+

(B)

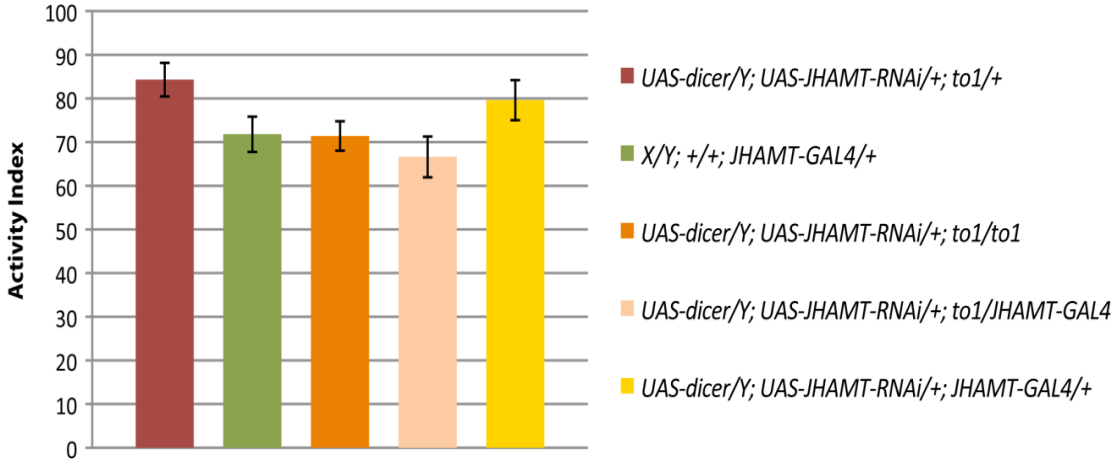

Activity Index
100
90
80
70
60
50
40
30
20
10
0
UAS-dicer/Y; UAS-JHAMT-RNAi/+; to1/+
X/Y; +/+; JHAMT-GAL4/+
UAS-dicer/Y; UAS-JHAMT-RNAi/+; to1/to1
UAS-dicer/Y; UAS-JHAMT-RNAi/+; to1/JHAMT-GAL4
UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+

Figure 26: Reduced Juvenile hormone levels do not genetically interact with a *takeout* heterozygous mutant state. (A) Courtship assay of the *takeout* mutant *UAS-dicer/Y; UAS-JHAMT-RNAi/+; to^1/to^1*, Juvenile hormone knockdown *UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+* and interaction *UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/to^1* flies. The genotype controls used were *X/Y; +/+; JHAMT-GAL4/+* and *UAS-dicer/Y; UAS-JHAMT-RNAi/+; to'/+*. The results do not appear to indicate a genetic interaction between *JHAMT* knockdown and a takeout heterozygous mutant background (n=10. Error bars represent s.e.m). (B) Activity assay of the above genotypes (n=10. Error bars represent s.e.m).

The experimental data do not appear to indicate a genetic interaction between reduction in Juvenile hormone levels and a *takeout* heterozygous mutant background (Figure 26). The courtship index shown by the *UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*, where Juvenile hormone synthesis is disrupted and the courtship index shown by *UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/to*^{1}, where Juvenile hormone is knocked down in a *takeout* heterozygous mutant background show no difference and was shown to be statistically insignificant. This could indicate that Juvenile hormone and *takeout* act in the same genetic pathway, or that there is still enough Takeout protein in the heterozygous flies for courtship function. The two control genotypes show a normal courtship index. *to*^{1} homozygous mutants show a reduced courtship index of 0.85, as has been previously described (Dauwalder, 2002). As shown previously, *to*^{1} */ +* heterozygous males do not have a courtship defect (Dauwalder, 2002).

It is possible that a further reduction in courtship index might be observed when Juvenile hormone levels are reduced by RNAi in a *takeout* homozygous mutant background. This experiment needs to be done in the future.

The activity assay performed on all the genotypes show that the activity indices of the courtship mutants are similar or higher than the indices of the two control genotypes. Therefore, this indicates that the courtship mutant phenotype is not a result of a locomotor defect.

3.3.7. Conditional ablation of the Corpora allata results in a courtship mutant phenotype

To further verify that the presence of optimal Juvenile hormone levels is vital for normal courtship behavior, directional cell ablation was performed on the Corpora allata using the

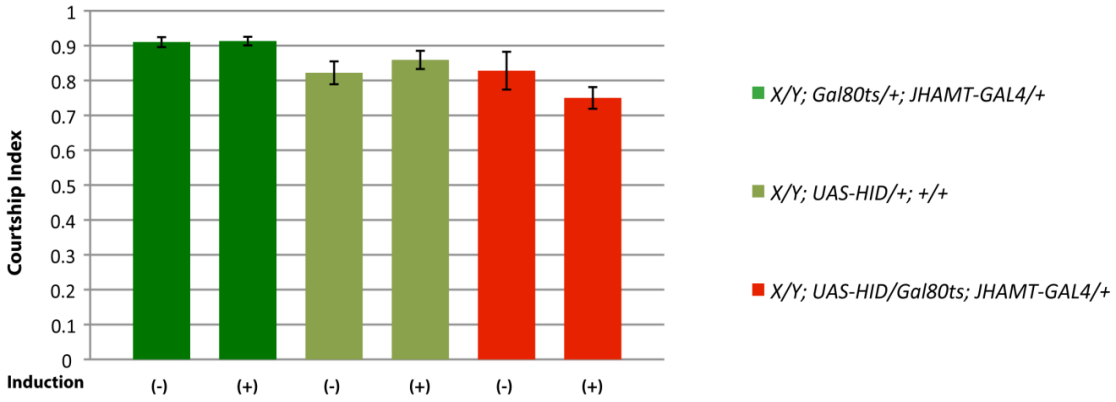
Corpora allata specific *JHAMT-GAL4* driver and two cell death gene constructs, *UAS-DTI* and *UAS-HID*.

3.3.7.1. *Cell ablation using the cell death gene construct UAS-HID*

The cell death construct *UAS-HID* has been previously used to ablate cells in the Corpora allata of the fly. The *UAS-HID* construct was used to ablate the Corpora allata together with a *UAS-reaper* cell death construct and resulted in a significant reduction in gland activity (Gruntenko et al., 2010; Gruntenko et al., 2012). The authors indicate that *JHAMT* activity, indicating the activity of the Corpora allata, is reduced in a *JHAMT* activity assay, when the cell ablation is performed. The authors claim that the use of the construct resulted in a partial reduction in the size of the organ.

Our Corpora allata specific *JHAMT-GAL4* driver was used to drive the expression of *UAS-HID* to ablate the Corpora allata cells. The experiment was carried out conditionally, using the $Gal80^{ts}$ system, in the adult flies. The experimental genotype was *X/Y; UAS-HID/* $Gal80^{ts}$*; JHAMT-GAL4/+,* where directional cell ablation was performed. The control genotypes were *X/Y; UAS-HID/ +; +/+* and *X/Y;* $Gal80^{ts}$*/+; JHAMT-GAL4/+,* containing the *UAS* and *JHAMT-GAL4* transgenes in two separate lines. These flies were created and raised at 18°C. The selected males were matured for 7 days at 18°C and induced at 32°C for 1 day. They were then kept at room temperature for 1 hour. The induced and un-induced experimental and control genotypes were subjected to a courtship assay.

(A)



(B)

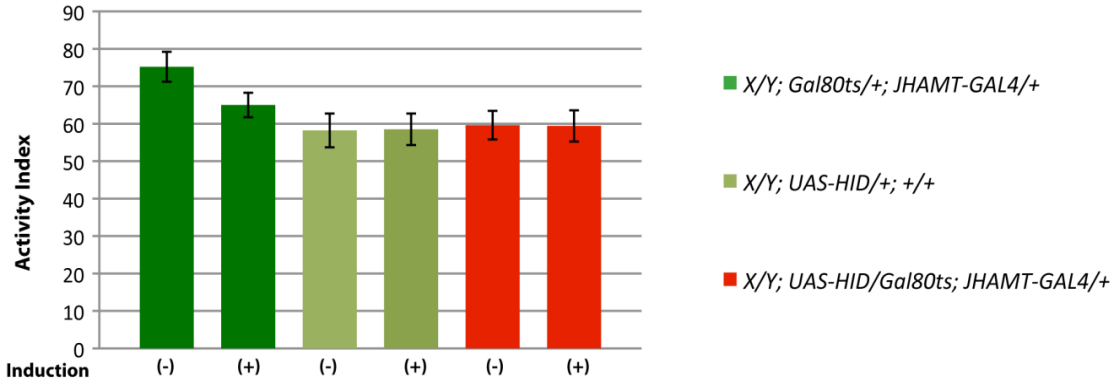


Figure 27: Expression of the cell death gene construct *UAS-HID*. (A) Courtship assay of induced and un-induced experimental *X/Y; UAS-HID/ Gal80^{ts}; JHAMT-GAL4/+* and control genotypes *X/Y; UAS-HID/ +; +/+* and *X/Y; Gal80^{ts}/+; JHAMT-GAL4/+* (n=15. Error bars represent s.e.m). (B) Activity assay of the above genotypes (n=10. Error bars represent s.e.m). (Induction; Un-induced= (-), Induced= (+)).

These results do not suggest a statistically significant reduction of courtship in males in which the HID cell death gene has been expressed (Figure 27) (p=0.05).The induced experimental genotype shows a reduction in courtship index to 0.75. The un-induced experimental genotype reduces the courtship index to 0.82, which could be due to a leaky *Gal80^{ts}*. The Corpora allata directed cell ablation system could be partially activated at 18 °C, partially ablating the Corpora allata at 18 °C and reducing Juvenile hormone levels. Further induction at 32^{o}C reduces the courtship index in addition. The control genotype *X/Y; Gal80^{ts}/+; JHAMT-GAL4/+* shows a >0.9 courtship index at the induced and un-induced states, which is considered a normal/ wildtype courtship index. However, the control genotype *X/Y; UAS-HID/ +; +/+*, wherein *UAS-HID* is present alone, indicates a 0.82 courtship index at the un-induced state and a 0.85 index at the induced state. This indicates a statistically significant effect of the *UAS-HID* transgene insertion itself on courtship.

As a result, statistical analysis using ANOVA does not indicate significance in the reduction of courtship between the induced experimental and induced and un-induced control genotypes.

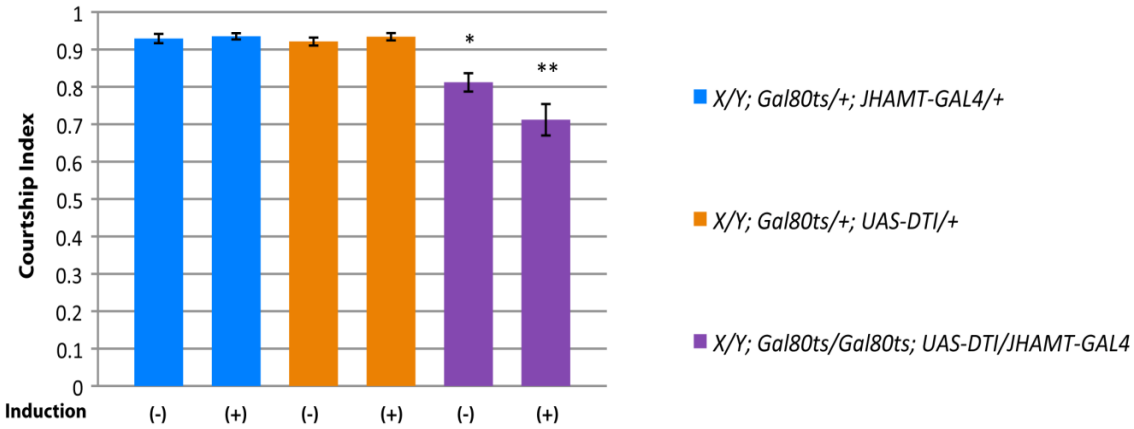
The activity indices of the induced and un-induced experimental flies are very comparable to that of the control genotype *X/Y; UAS-HID/; +/+*.

3.3.7.2. *Cell ablation using the cell death gene construct UAS-DTI results in a courtship mutant phenotype*

We therefore sought another method to ablate the Corpora allata in adult males. We used the protein synthesis inhibitor gene DTA to ablate the Corpora allata. As the toxicity of DTA is extreme, an attenuated mutant, DTI is used. In the current study, the *UAS-DTI* driven cell ablation was carried out conditionally in adult flies, using the *Gal80^{ts}* system. The experimental

genotype was *X/Y; Gal80^{ts}/ Gal80^{ts}; UAS-DTI/JHAMT-GAL4*. The control genotypes were *X/Y; Gal80^{ts}/+, JHAMT-GAL4/+* and *X/Y; Gal80^{ts}/+; UAS-DTI/+*, placing the *JHAMT-GAL4* and *UAS* constructs in two separate fly lines. The flies were created and maintained at 18°C. The selected males were matured for 5 days at 18°C and placed in a 30°C incubator for 2 days for induction. The flies were then kept at room temperature for one day. The induced and un-induced experimental and control genotypes were subjected to a courtship assay. The assay was done to 20 replicates.

(A)


Courtship Index
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
Induction
(-)
(+)
(-)
(+)
(-)
(+)
X/Y; Gal80ts/+; JHAMT-GAL4/+
X/Y; Gal80ts/+; UAS-DTI/+
X/Y; Gal80ts/Gal80ts; UAS-DTI/JHAMT-GAL4

(B)

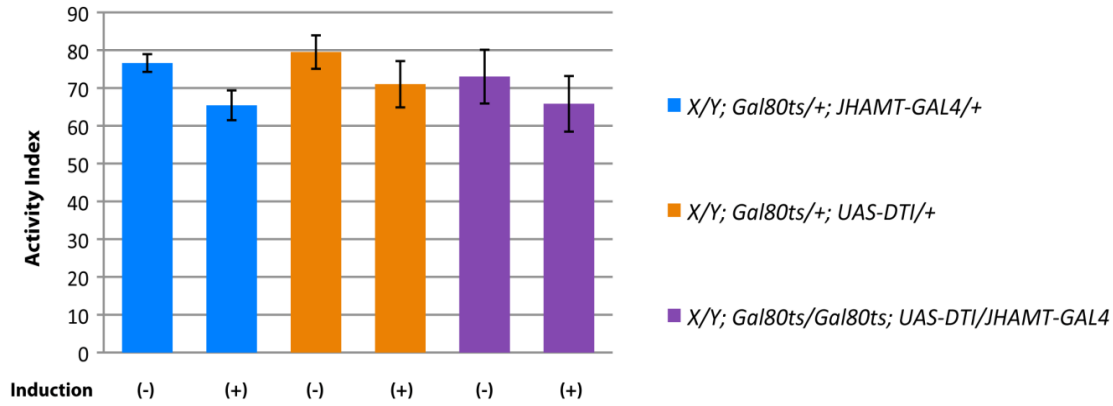

Activity Index
90
80
70
60
50
40
30
20
10
0
Induction
(-)
(+)
(-)
(+)
(-)
(+)
X/Y; Gal80ts/+; JHAMT-GAL4/+
X/Y; Gal80ts/+; UAS-DTI/+
X/Y; Gal80ts/Gal80ts; UAS-DTI/JHAMT-GAL4

Figure 28: Cell ablation using the cell death gene construct *UAS-DTI* results in a courtship mutant phenotype. (A) Courtship assay of the Corpora allata ablated flies and control flies. Experimental genotype *X/Y; Gal80^{ts}/ Gal80^{ts}; UAS-DTI/JHAMT-GAL4* and control genotypes *X/Y; Gal80^{ts}/+; JHAMT-GAL4/+* and *X/Y; Gal80^{ts}/+; UAS-DTI/+*, induced and un-induced were assayed. The experimental genotype shows a significant reduction in courtship index. The experimental genotype when un-induced, shows a significant reduction in courtship index compared to the control genotypes as indicated by asterix *(p=0.0005). The experimental genotype shows a further reduction in courtship index at the induced state when compared to the controls, which is shown to be significant as indicated by asterix ** (p<0001) (n=20. Error bars represent s.e.m). (B) Activity assays of the above genotypes. Activity assays indicate that the experimental induced condition is comparable to one of the controls (induced *X/Y; Gal80/+; JHAMT-GAL4/+*) and is therefore not considered defective in locomotion (n=10. Error bars represent s.e.m). (Induction; Un-induced= (-), Induced= (+)).

This experiment is the first instance that the *UAS-DTI* cell death construct has been utilized to achieve genetic ablation of the Corpora allata. The experiment clearly indicates that the genetic ablation of the Corpora allata results in a courtship mutant phenotype (Figure 28). The induced experimental genotype gives a courtship index of 0.71 which was shown to be statistically significant compared to the un-induced (p=0.0014). The un-induced experimental genotype also did show a courtship reduction, when compared to the controls the index was reduced to 0.81. This reduction was also shown to be statistically significant (p< 0.0005). This could be due to a 'leaky' *Gal80^{ts}*. The *UAS-DTI* construct might be activated by the Corpora allata specific *JHAMT-GAL4* driver in the un-induced state. This experimental phenotype contained two copies of *Gal80^{ts}*. Therefore, even in the presence of two copies of *Gal80^{ts}*, there is some leakiness. This indicates that the *UAS-DTI* construct is very toxic to cells even at very low levels. The control genotypes *X/Y; Gal80^{ts}/+; JHAMT-GAL4/+* and *X/Y; Gal80^{ts}/+; UAS-DTI/+* did show a >0.9 courtship index in the induced and un-induced states, indicating that the *UAS-DTI* construct was activated only in the presence of the driver, the construct alone, does not result in a courtship deficiency.

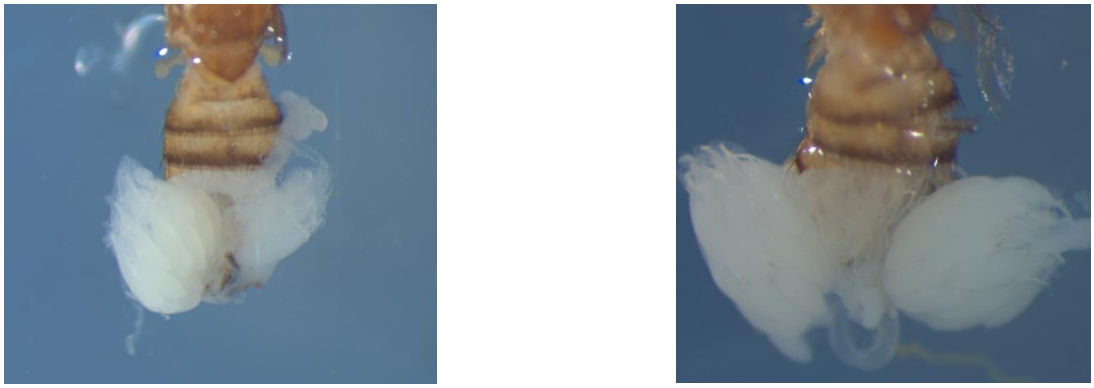
3.4. Verification of Juvenile hormone synthesis disruption

3.4.1. Verification by defective ovary development

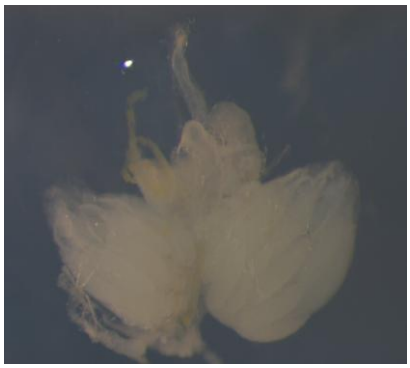
Previous research has shown that optimal levels of Juvenile hormone are required for proper development of ovaries in female insects (Dubrovsky et al., 2002; Soller et al., 1999). Therefore,

this phenotype was used to verify the knockdown of Juvenile hormone levels in the adults by RNAi against *JHAMT*.

The genotypes analyzed for reduction in Juvenile hormone levels were those in which the disruption of Juvenile hormone synthesis was carried out throughout development. The experimental genotype was *UAS-dicer/X; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*, in which Juvenile hormone levels are expected to be reduced. The control genotypes were *UAS-dicer/X; UAS-JHAMT-RNAi/+; +/+* and *X/X; +/+; JHAMT-GAL4*. The flies were created and raised as for the behavior experiment. The selected virgin female flies were matured for 3 days for ovary development and dissected to expose the ovaries. 10 flies per genotype were examined. They were examined using a Zeiss microscope and photographed.



(A) **(B)**



(C)

Figure 29: Disruption of Juvenile hormone synthesis in the Corpora allata by directed RNAi is verified by defective ovary development in females. (A) The experimental genotype *UAS-dicer/X; UAS-JHAMT-RNAi/+; UAS-JHAMT-GAL4* ovaries. (B) Control genotype *UAS-dicer/X; UAS-JHAMT-RNAi/+; +/+* ovaries. (C) Control genotype *X/X; +/+; JHAMT-GAL4* ovaries.

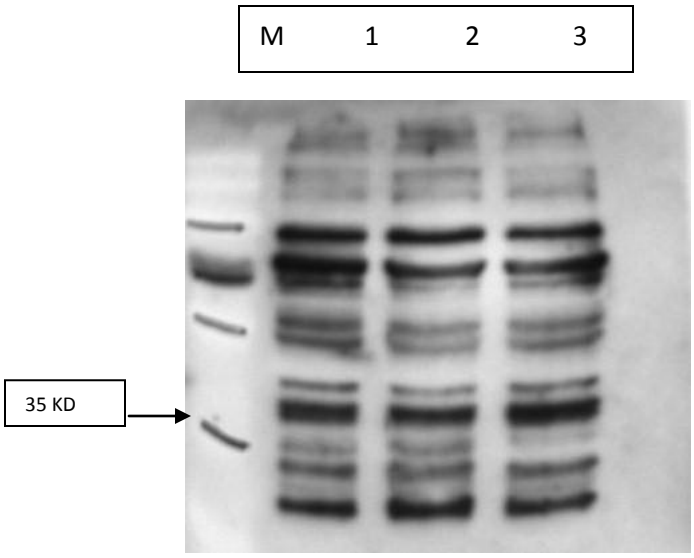
The images are consistent with a reduction of Juvenile hormone synthesis, resulting in ovary defects. The control genotypes *X/X; +/+; JHAMT-GAL4* and *UAS-dicer/X; UAS-JHAMT-RNAi/+; +/+* show a normal size of the ovaries, while the experimental genotype *UAS-dicer/X; UAS-JHAMT-RNAi/+; UAS-JHAMT-GAL4/+* show ovary defects. The mutant females show reduced egg production in one ovary compared to the other (Figure 29). This results in the defective ovary being reduced in size. Both ovaries of females of the control genotypes are of similar size. Ten females were dissected and examined per genotype. The mutant phenotype was not uniformly observed in all flies of the experimental genotype. Some flies (3/10) didn't clearly display defective ovary development. This could be due to a weak knockdown in Juvenile hormone levels in the females.. The RNAi knockdown of Juvenile hormone levels by the driver and *UAS-JHAMT-RNAi* constructs that were used could be insufficient to reduce Juvenile hormone titers in the female flies to a level that shows a uniform effect on ovary development. The RNAi knockdown that was used was also insufficient to affect the full development of the flies, indicating a weak knockdown.

In summary, these results suggest that expression of *JHAMT-RNAi* by *JHAMT-GAL4* results in lower Juvenile hormone levels.

3.4.2. Examination by immunoblotting

To verify the reduction of *JHAMT* levels by RNAi, the expression of the enzyme in the flies was examined by Western blotting. The genotypes used for the experiment were those that were used for the initial behavior experiment, in which the knockdown of *JHAMT* levels by RNAi was carried out throughout development. Specifically, the experimental genotype was *UAS-dicer/Y;*

UAS-JHAMT-RNAi/+; UAS-JHAMT-GAL4/+. The control genotypes were *X/Y; +/+; JHAMT-GAL4* and *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+*. Protein was extracted from whole flies. The membrane was stained using rabbit raised α-JHAMT as the primary antibody. The antibody is an anti-serum raised against the *Dm*JHAMT protein in rabbit. (Niwa et al., 2008). This antibody was previously used in Western blots to detect the expression of a recombinant *Dm*JHAMT expressed in *E.coli* (Niwa et al., 2008). The results are shown in Figure 30.



M. Pre-stained marker

1. Treatment

(*UAS-dicer/Y; UAS-JHAMT-RNAi/+; UAS-JHAMT-GAL4/+*)

2. Control 1 (*X/Y; +/+; JHAMT-GAL4/+*)

3. Control 2 (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+*)

Figure 30: Examination of JHAMT knockdown at room temperature using immunoblotting.

Immunoblotting was performed on protein extracts from Juvenile hormone knockdown (*UAS-dicer/Y; UAS-JHAMT-RNAi/+; UAS-JHAMT-GAL4/+*) and control flies (*X/Y; +/+; JHAMT-GAL4/+* and *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+*). The blot was stained with a α-JHAMT antibody at 1:250 dilution and a secondary HRP-α-rabbit antibody at 1:10,000 dilution. 0.1 OD of protein extracts were loaded per lane. The expected position of the JHAMT protein (35 KD) is indicated by arrow.

The Western blot does not indicate a reduction in protein levels of JHAMT in the knockdown flies when compared to the controls (Figure 30). Since there are a number of unspecific bands, without optimization of the Western blot we cannot draw a conclusion from this experiment. The antibody is an un-purified anti-serum raised in rabbit against JHAMT. Additionally, the anti-serum was used in the previous research to stain against a recombinant protein expressed in *E. Coli* (Niwa et al., 2008). But, in the current study, the anti-serum was used to stain against protein extracts from whole flies. Therefore, the specificity could be reduced.

Behavior experiments on the above flies indicated a reduction in courtship after *JHAMT* knockdown by RNAi (Figures 20, 22-24, 26). This phenotype was also rescued by application of Methoprene to flies after knockdown of Juvenile hormone levels (Figures 21, 25). These observations indicate the reduction of Juvenile hormone levels in the flies by the RNAi treatment. The knockdown in JHAMT protein levels may not be apparent as the levels of *JHAMT* knockdown are not sufficient to be detected by Western blotting. However, even a slight level of JHAMT knockdown could reduce the levels of Juvenile hormone sufficiently to impact the courtship behavior, which was also rescued by application of the Juvenile hormone analog, Methoprene. The reduced level of knockdown of *JHAMT* could be due to a reduced strength of the *JHAMT* promoter or the *UAS-JHAMT-RNAi* construct. The α-JHAMT antibody observed to be less specific as it appears to stain many proteins to give many bands.

3.4.3. Examination of *JHAMT* knockdown using qPCR

Since the Western blotting experiment for the JHAMT protein was not specific and therefore inconclusive, we used qPCR as another approach to examine RNAi mediated reduction of *JHAMT*.

3.4.3.1. *JHAMT* knockdown at room temperature

RNA extracts of whole males were used. RNA was prepared from *UAS-dicer/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+* and the control genotypes *X/Y; +/+; JHAMT-GAL4/+* and *UAS-dicer/Y; UAS-JHAMT-RNAi/+; +/+* males.

The flies were raised as for the behavior experiment. They were isolated and matured for 4 days, frozen on dry ice and kept at -80°C. RNA was extracted from flies using the Trizol method and cDNA synthesized. A TaqMan *JHAMT* probe was used for qPCR analysis as described in Material and Methods.

The experiment was carried out for three biological replicates. The average deltaCT fold change value was calculated for all three replicates and plotted against the genotype. A standard curve was established with wildtype CS flies.

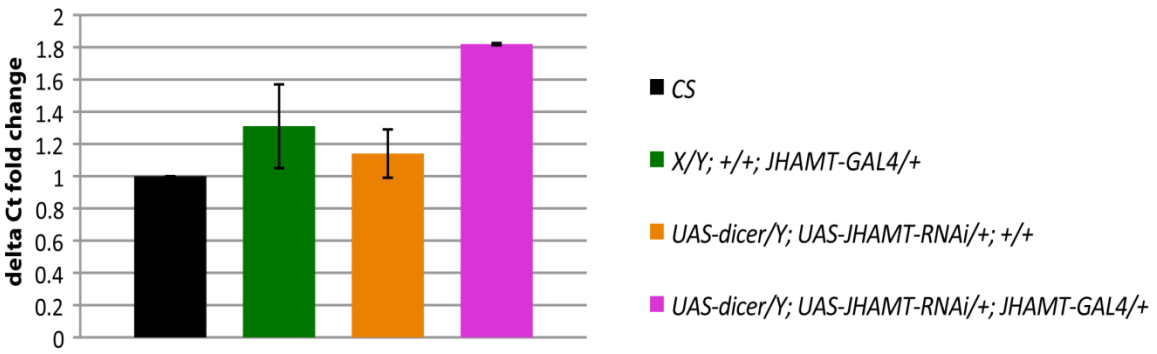


Figure 31: Examination of *JHAMT* knockdown at room temperature, using qPCR. qPCR was performed on three biological replicates of *JHAMT* knockdown flies in which knockdown was performed throughout development. The fold change of *JHAMT* expression and endogenous control rp49 of three technical replicates from whole flies for biological replicates of each genotype were averaged and plotted against the genotype. The results do not indicate a knockdown of *JHAMT* levels by RNAi (n=3).

The results of quantifying the mRNA knockdown using qPCR do not show a reduction of *JHAMT* levels in the flies. For a predicted standard Ct value of endogenous rp49 per dilution of each genotype, the ΔCt fold change value is expected to be lower if the expression of the gene is lower. However, the treatment knockdown genotypes appear to indicate a higher value (Figure 31).

3.4.3.2. Conditional knockdown of *JHAMT* by *hsp70-GAL4* and *UAS-JHAMT-RNAi*

Quantitative PCR was performed on induced and un-induced flies of the genotypes used for *hsp70-GAL4* induced RNAi of *JHAMT*. The flies were prepared as for the behavior experiments. The flies of experimental (*X/Y; UAS-JHAMT-RNAi/+; JHAMT-GAL4/+*) and control genotypes (*X/Y; UAS-JHAMT-RNAi/+; +/+* and *X/Y; +/+; JHAMT-GAL4/+*) were raised at room temperature. They were then matured for 4 days at room temperature and induced for 1 hour at 37°C. The flies were kept to rest for 4 hours and frozen at -80°C. RNA was extracted from the flies and quantitative PCR performed as mentioned in Materials and Methods.

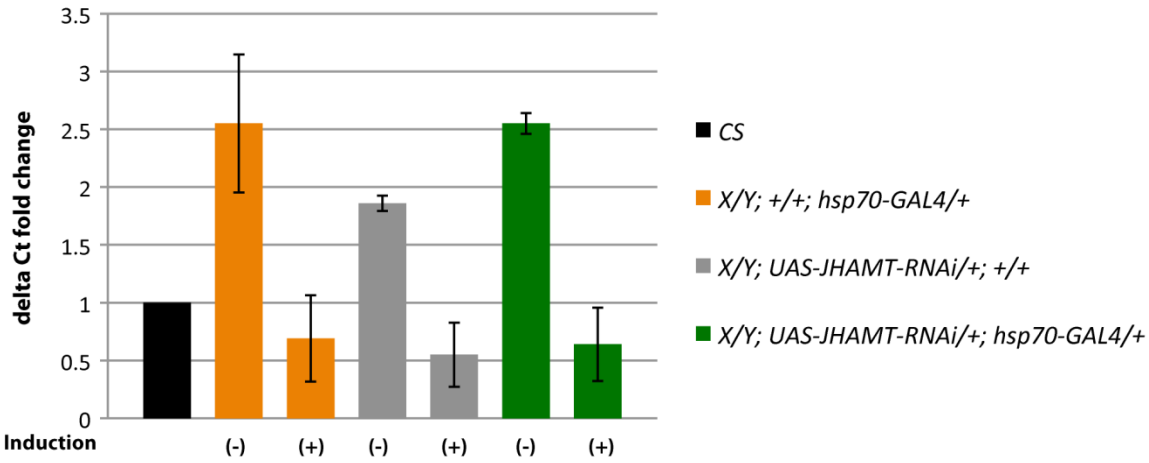


Figure 32: Examination of *JHAMT* knockdown by *hsp70-GAL4* driven *UAS-JHAMT-RNAi* using qPCR. qPCR was performed on induced and un-induced flies of genotypes that were used for the *hsp70-GAL4* driven RNAi of *JHAMT*. The relative expressions of *JHAMT* and endogenous control rp49 of mRNA from whole flies for each genotype were plotted against the genotype. The results do not indicate a clear reduction in *JHAMT* mRNA levels by RNAi at the experimental induced state. A reduction in *JHAMT* mRNA levels is indicated in all genotypes in response to heat shock (n=3). (Induction; Un-induced= (-), Induced= (+)).

The qPCR experiment indicates a reduction in expression after induction in all the genotypes tested (Figure 32), i.e. the experimental (*X/Y; UAS-JHAMT-RNAi/+; hsp70-GAL4/+*) and control genotypes (*X/Y; UAS-JHAMT-RNAi/+; +/+* and *X/Y; +/+; hsp70-GAL4/+*). This could be a general shock response to heat. The heat level used to activate the *hsp70-GAL4* driver is comparatively higher than that used for other experiments (e.g. $Gal80^{ts}$ experiments). Furthermore, under induced conditions, the experimental genotype does not appear to indicate a reduction in *JHAMT* levels when compared to the controls. The three genotypes appear mostly comparable. The basal levels of *JHAMT* mRNA (i.e. the un-induced states) levels also seem to show a high variation between genotypes, which complicates the analysis of knockdown.

3.4.3.3. Conditional knockdown of *JHAMT* by *JHAMT-GAL4* and *UAS-DTI*

qPCR was performed on induced and un-induced flies of the genotypes used for *JHAMT-GAL4* induced Corpora allata ablation using the *UAS-DTI* construct. The flies were prepared as for the behavior experiments. The flies of experimental (*X/Y;* $Gal80^{ts}/Gal80^{ts}$*; JHAMT-GAL4/UAS-DTI*) and control genotypes (*X/Y;* $Gal80^{ts}$*/+; JHAMT-GAL4/+* and *X/Y;* $Gal80^{ts}$*/+; UAS-DTI/+*) were raised at 18°C. They were then matured for 7 days at room temperature and induced for 2 days at 30°C. The flies were kept to rest for 1 day and frozen at -80°C. RNA was extracted from the flies and quantitative PCR performed as mentioned in Materials and Methods.

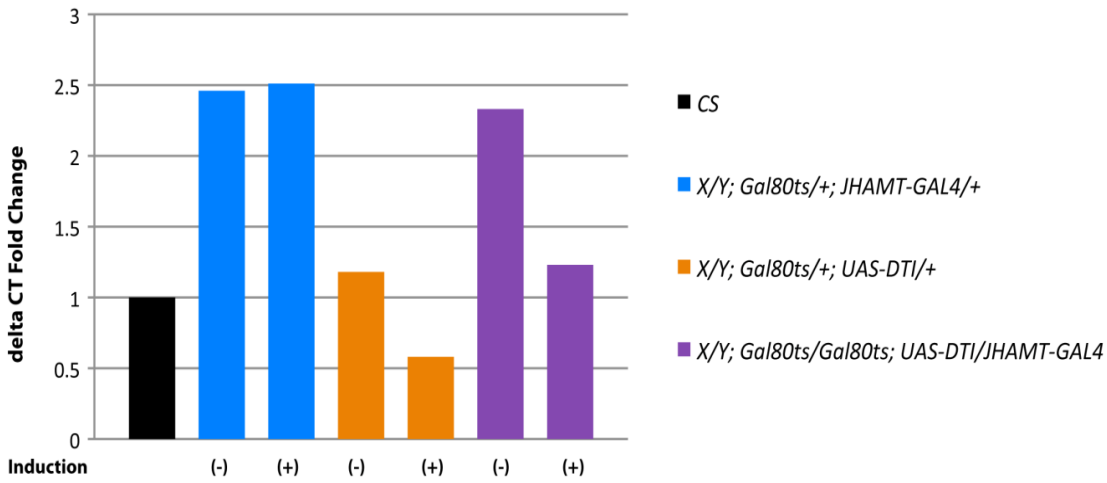


Figure 33: Examination of *JHAMT* reduction by genetic ablation of the Corpora allata by *JHAMT-GAL4* driven *UAS-DTI* using qPCR. qPCR was performed on induced and un-induced experimental and control genotypes used for genetic ablation of the Corpora allata. The fold change of the Ct values (Threshold values) of *JHAMT* and endogenous control rp49 of cDNA from whole flies for each genotype were plotted against the genotype (n=1). (Induction; Un-induced= (-), Induced= (+)).

The qPCR performed on induced and un-induced flies used for the genetic ablation of Corpora allata using *UAS-DTI* construct driven by *JHAMT-GAL4* promotor indicates a reduction in mRNA levels in the experimental genotype (*X/Y; Gal80^{ts}/Gal80^{ts}; JHAMT-GAL4/UAS-DTI*) and the control genotype containing the *UAS-DTI* construct (*X/Y; Gal80^{ts} /+; UAS-DTI/+*) when induced in comparison with the un-induced state (Figure 33). The control genotype containing the *JHAMT-GAL4* construct does not show clear reduction in expression when flies are induced. The reduction in mRNA levels (ΔCt fold change) from induced to un-induced is higher in experimental genotype when compared to the control, which could indicate a reduction in *JHAMT* mRNA levels with the ablation of the Corpora allata. When the *UAS-DTI* construct is present, the *JHAMT* levels also show a reduction when induced, which could be attributed to construct being active without a *GAL4* driver at high temperatures. This could be causing unspecific cell ablation within the fly. However, it is not possible to form a definite conclusion based on one replicate. Multiple replicates are required to examine for statistical significance.

In summary, we did not observe a reduction in *JHAMT* RNA levels. This is despite the fact that the courtship mutant phenotype in *JHAMT* knockdown flies could be rescued by application of Juvenile hormone analog, Methoprene, and that we have observed an effect of *JHAMT* RNAi expression on ovaries. This discrepancy and the qPCR results are discussed in detail in the discussion.

CHAPTER IV

RESULTS 2

CHAPTER IV: RESULTS 2. BINDING BETWEEN TAKEOUT AND JUVENILE HORMONE

As mentioned in the introduction and shown in Figure 7 B, Takeout has its highest sequence similarity with JHBPs. I therefore performed experiments to test the hypothesis that Takeout binds Juvenile hormone. I produced Takeout-GST protein in a Baculovirus expression system, and purified it using GST purification. I subsequently processed the protein to remove the GST tag and used it for binding studies. Binding to JH^{H3} was tested using equilibrium dialysis.

4.1. Takeout expression using the Baculovirus expression system

4.1.1. Overview

We chose to express the Takeout protein in a Baculovirus expression system rather than in E. coli, because this system uses Sf9 insect cells for amplification of the virus and production of protein. In these cells, eukaryotic protein modifications will occur. It was our goal to produce Takeout protein that would be as similar as possible to the *in vivo* produced fly protein.

The *takeout* protein-coding sequence that corresponds to the secreted protein (and thus does not contain the *takeout* secretion signal sequence) (Figure 14) [Appendix 2 A] was amplified from cDNA and cloned into an intermediate pSC-B vector. The sequence was verified by sequencing, removed from the vector using restriction enzymes and cloned into the pAcSecG2T vector (Figure 15)[Appendix 2 D], in frame with a vector-encoded signal peptide that is designed for secretion of the recombinant protein into the cell medium. The *takeout* sequence was

further placed downstream of a vector-encoded GST sequence [Appendix 2 D]. This resulted in the expression of a Takeout-GST fusion protein. The GST tag allows purification of the fusion protein from the culture medium using the GST purification system.

The pAcSecG2T-*takeout* construct was recombined with virus sequences using the BaculoGold® Baculovirus system (BD Biosciences) and transformed into Sf9 cells following the manufacturer's protocol. The recombinant virus was amplified in Sf9 cells several times to increase viral titer. The virus stock was then used to express the Takeout-GST fusion protein in Sf9 cells. Initial expression was performed in Sf9 cells growing in serum-containing medium. The Takeout-GST fusion protein expression obtained was very low, suggesting that the virus titer might be low, or that expression might not be optimal in this Sf9 cell line. In order to optimize expression and produce protein, our virus stock was sent to the company Allele Biotechnology, CA. They amplified our virus stock to produce a stock with a higher titer. Expression in three different cell lines, Tni, Sf9 and Sf9 super cells was performed and we determined that Tni cells that grow in serum-free media gave the best expression of the protein. Consequently, Tni cells were used for further expression of the protein. Expression in the serum-free media has the additional advantage that expressed Takeout protein is not obscured by serum proteins of similar size when visualized on a protein gel. The resulting protein was purified using a GST purification column and returned back to us.

4.1.2. Creating the Recombinant BaculoGold-*takeout-GST* virus

4.1.2.1. Creating the pAcSecG2T-*takeout* construct

The *takeout* sequence was amplified from *takeout* cDNA using takeout primers and the Phusion high fidelity polymerase and two step PCR. The expected size of the product is 758 bp (Figure 34 A).

PCR reactions 1 and 6 were used for cloning.

Both of the above samples containing the *takeout* sequence were cloned into the pSC-B PCR blunt cloning vector following the manufacturer's protocol. The construct was transformed into E.Coli cells, colonies selected and DNA preps prepared using the QIAGEN mini-prep method. The cloning was confirmed by digestion with Sma-1 (Figure 34 B).

Mini-preps No. 1 of each clone (clone 1 and 6) (Figure 34 B) was chosen and sequenced for sequence verification.

Next**,** the *takeout* insert was cloned into the pAcSecG2T vector (Figure 35). The recipient vector, pAcSecG2T, and pSC-B- *takeout* were digested with Xma-1, run on a gel and extracted.

The pAcSecG2T vector and *takeout* were ligated with a vector to insert ratio of 1:3 and transformed into NEB competent cells. Mini preps of resulting colonies were test digested using Xma-1 and examined on a 1% agarose gel.

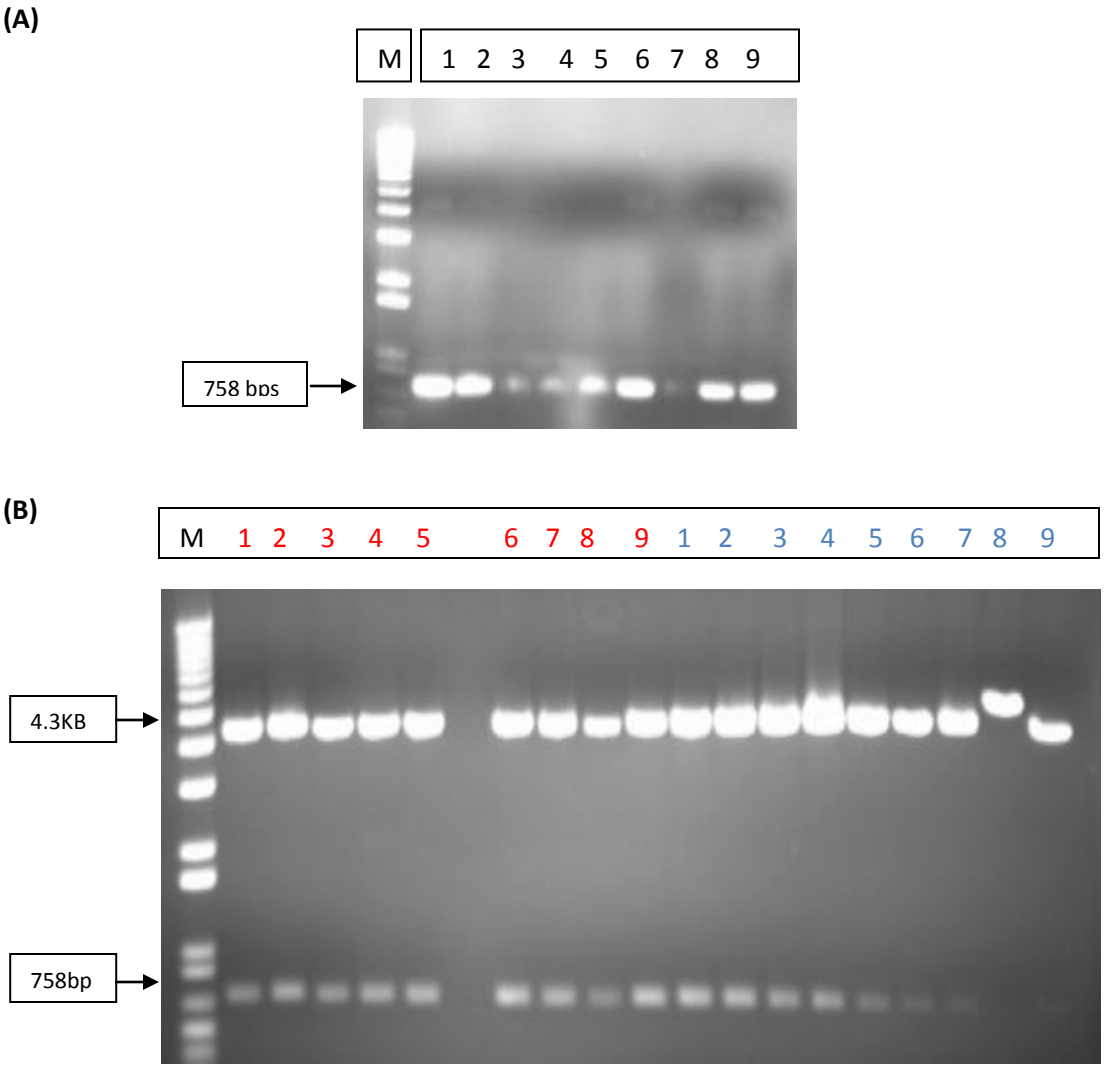


Figure 34: *takeout* sequence amplification from cDNA and cloning into pSC-B. (A)The *takeout* sequence as amplified from cDNA using two step PCR. The *takeout* sequence without the signal peptide sequence has a size of 758 bps. (B) Restriction enzyme test digest of pSC-B+ *takeout* constructs using the Sma-1 restriction enzyme. Arrows indicate the position of the 4.3 kb vector and the *takeout* insert, respectively. All of the clones show the presence of the 758 bp insert released from the 4.3 Kb vector. Restriction test digests of Mini-preps of PCR reaction 1 are labeled in red, while those of PCR reaction 2 are labeled in blue.

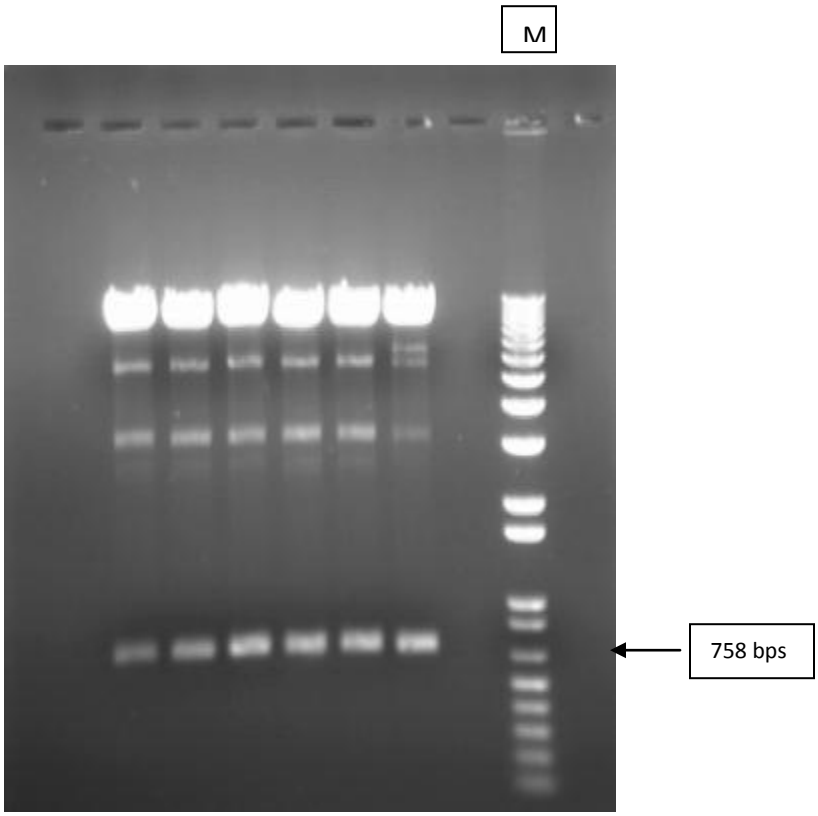
[The pAcSecG2T-*takeout* construct is displayed in Appendix 2 D]

Restriction enzyme digestion, using Xma-1 was carried out on mini-prep samples of the construct to verify the presence of the *takeout* insert (Figure 35 B).

The mini-prep samples containing the construct were then test digested using BamH1, to further verify the orientation of the insert (Figure 35 B).

The correct orientation of the insert should result in a 90 bp fragment of DNA along with an 8551 bp band. Colony 09 with the correct orientation was chosen for further experiments.

(A)



(B)

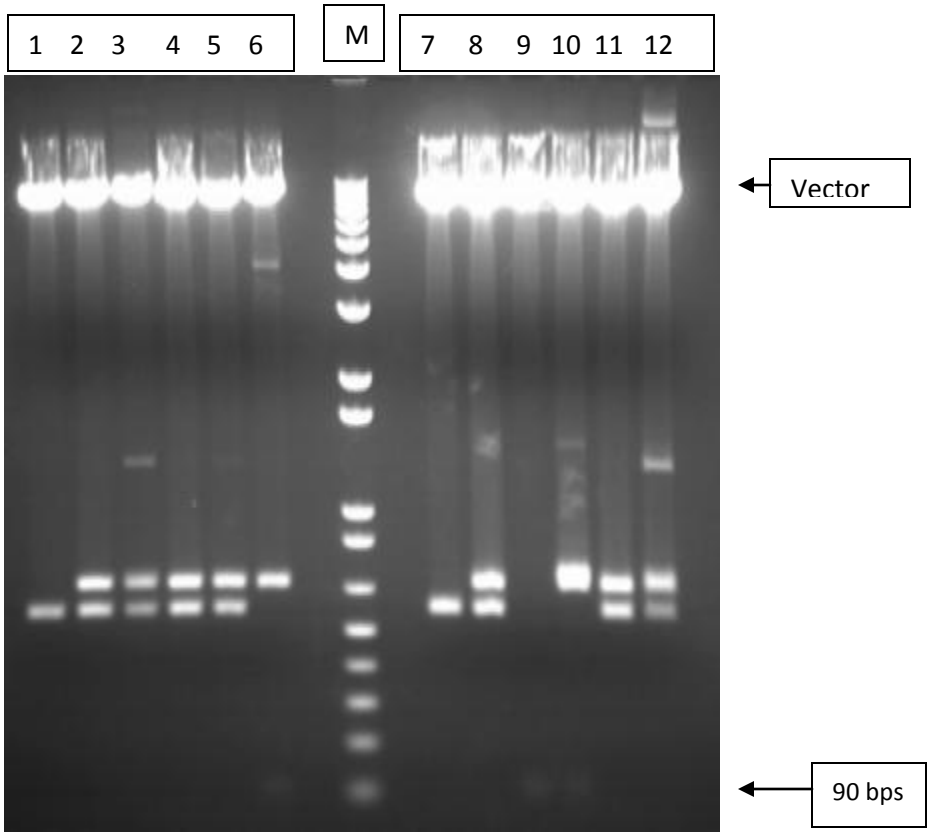


Figure 35: Cloning of the *takeout* sequence into the recipient pAcSecG2T vector and restriction enzyme verification of the presence and orientation of the *takeout* insert in the vector. (A) Restriction enzyme test digest of pAcSecG2T-*takeout* construct. The construct was digested using Xma-1 restriction enzyme to verify the presence of the *takeout* sequence. The 758 bp sequence was release from the vector. (B) The construct was digested with BamH1 restriction enzyme to verify the direction of the *takeout* sequence in the construct. The correct orientation should cleave off a 90 bp fragment.

4.1.2.2. Recombination between pAcSecG2T-*takeout* construct and BD BaculoGold

The pAcSecG2T vector containing the *takeout* sequence was co-transfected with BD BaculoGold virus into Sf9 cells in TNM-FH medium following the supplier's protocol. The pAcSecG2T vector alone was co-transfected into Sf9 cells with BaculoGold DNA as transfection control. The cells were maintained at 28°C for 4 days. After 4 days, the cells appeared burst and the culture contained cell debris, indicating infection by the virus. The viral supernatant was extracted from the culture and amplified 2-3 times by re-transfecting the Sf9 cells and incubating at 28°C. The resulting amplified virus was used to transfect Sf9 cells in TNM-FH medium.

4.1.3. Protein expression in Sf9 cells in TNM-FH medium

The amplified virus from the 3rd amplification was used to express the Takeout-GST protein in Sf9 cells in serum containing culture. The cells were infected with 500µl and 5ml of viral stock solution in two independent experiments. The protein expression was analyzed after 3 days of culture. The *takeout-GST* sequence in the pAcSecG2T vector is preceded by a secretion signal. Therefore, the fusion protein was expected to be secreted from the cells resulting in the major portion of the protein being present in the culture medium. There was also a possibility that the protein, although expressed in the cells, would not be secreted, resulting in accumulation in the cell fraction. Therefore, the culture medium and the cell fractions were analyzed for presence of the Takeout-GST fusion protein using immunoblotting. The Takeout-GST fusion protein and GST only protein bands were detected using α-GST primary antibody and α-mouse secondary antibody.

When analyzed for protein expression, the GST protein expressed by the control BaculoGold-*GST* virus was present while the Takeout-GST fusion protein expressed by BaculoGold-*takeout-GST* virus was absent when infected with either 500µl or 5 ml of viral supernatant. In the control, the GST protein was present in both the supernatant and the cell pellet. It is possible that the Takeout-GST fusion protein was not expressed by the virus. The infection of the cells in culture by the virus was previously observed, thereby eliminating the possibility that the virus did not infect the cells. The Baculovirus expression system did express the GST protein from the Baculovirus pAcSecG2T recombined virus, indicating that the recombination between the vector and the virus as well as expression of protein from the recombinant virus after infection of the cells are possible. The Takeout-GST fusion protein could be absent due to the absence of recombination between virus and vector or because of the absence of expression from the recombined virus. The fusion protein was absent in both the supernatant and the cell pellet.

GST purification was tested on the Baculovirus-*takeout-GST* infected samples and Baculovirus-*GST* infected samples. It was expected that as GST purification of the samples would concentrate them, if Takeout-GST is present in the culture medium, it would be concentrated and therefore could be visible. The protein culture medium of the above experiment in which 5ml of virus was used to infect the cells was analyzed. The purification was done using Pierce Glutathione GST-purification mini-columns following the manufacturer's protocol.

The three elution fractions of the GST purification procedure were analyzed by Western blot for the presence of GST. These Western blots showed the presence of GST protein in the control, but did not show any Takeout-GST protein in the experimental (Figure 36). This demonstrates

that GST is capable of binding to the Glutathione agarose column and eluting from the column upon addition of Glutathione.

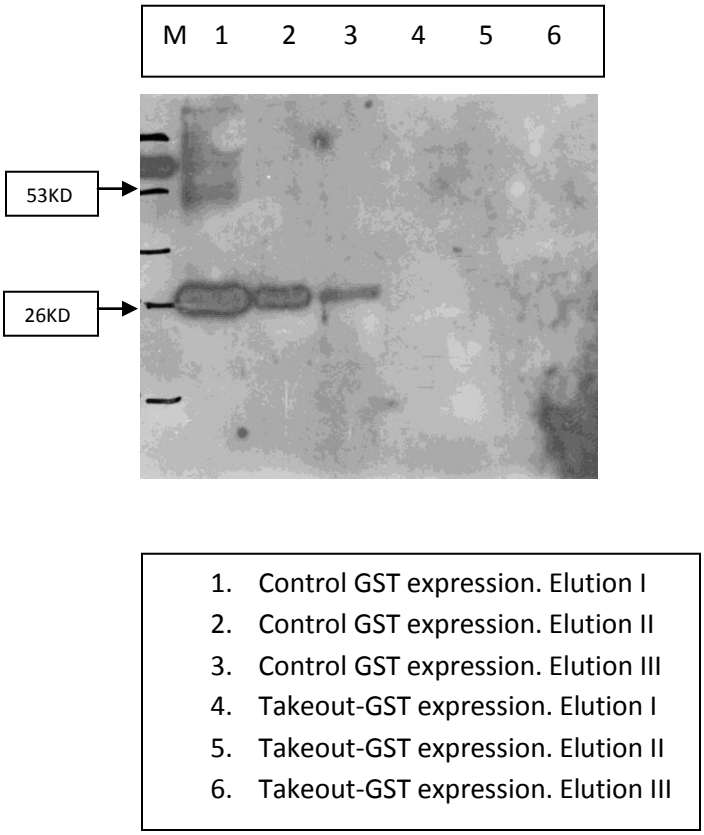


Figure 36: BaculoGold-*takeout-GST* (experimental) infected Sf9 cell cultures does not show expression of Takeout-GST fusion protein. The cell cultures were infected with 5ml of the respective recombinant viruses. The cultures were GST purified after protein expression. 20µl (of 1 ml culture samples GST purified to 50µl) each of the three fractions of purification (I, II and III) were analyzed by anti-GST Western blotting. The figure shows the expression and successful purification of the GST protein (26KD) from the control culture, but not the Takeout-GST fusion (53KD) protein from the experimental culture.

The Takeout-GST protein was apparently not expressed in the above experiment (Figure 36). There could be several possibilities for absence of expression. A time course experiment was performed to examine if there exists an optimal time point for expression of the protein and/ or if there is any degradation of the protein in the culture medium. The Sf9 cells in serum containing TNM-FH medium were infected with 5ml of the recombinant virus stock. The experiment was done using the experimental Baculovirus-*takeout-GST* virus and the control Baculovirus-*GST* virus in two separate experiments. The 3rd amplification of the virus was used. The cell and culture medium fractions were analyzed for the expression of the protein after 6, 15, 24, 48 and 72 hours of culture. 1ml samples (of 15ml total) of culture medium fractions were concentrated and analyzed. Cell pellet samples were lysed to release the protein and analyzed. The analysis did not indicate the presence of Takeout-GST in the BaculoGold-*takeout-GST* infected experimental culture while it did show the presence of the GST protein in the BaculoGold-GST infected control culture in all time points of expression. The GST protein was present in the culture (cells and culture medium) at all time points. Therefore, this indicates absence of Takeout-GST expression by the experimental BaculoGold-*takeout-GST* virus.

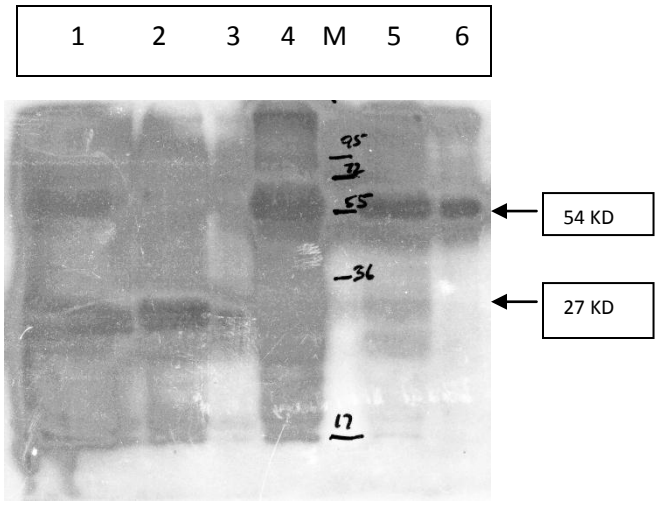
The previous protein expression experiments do not show the presence of the Takeout-GST protein in culture. The control BaculoGold-*GST* infected culture expressed a GST protein that can be fully purified by GST purification. This control acts as a transfection control indicating the ability to transfect the recombinant BaculoGold-*GST* virus into Sf9 cells and express the GST protein. The absence of the Takeout-GST protein in the experimental BaculoGold-*takeout-GST* infected culture could be an indication of absence of recombination between BaculoGold virus and pAcSecG2T-*takeout* construct. Other possibilities would be that the recombinant

BaculoGold+*takeout-GST* virus did not transfect the Sf9 cells and/or that there was a defect in the virus that prevented protein expression.

Therefore, creation of the recombinant BaculoGold+*takeout-GST* virus was repeated. The BaculoGold-*GST* was also created to use as a control. The creation of the two recombinant viruses was carried out as done previously. They were subsequently used to transfect Sf9 cells in TNM-FH medium and protein was harvested from the cultures after 3 days. The culture medium and cell fractions were analyzed using immunoblotting. The *GST* only expression cultures (control) and *takeout-GST* expression cultures were subjected to PAGE at different dilutions of protein sample. The culture medium was concentrated using Millipore concentrators. 1 ml of medium was concentrated to 50µl and subsequently diluted to 0.1, 0.01, and 0.001. The cell pellet was dissolved in EB-2 protein buffer and diluted to the above concentrations.

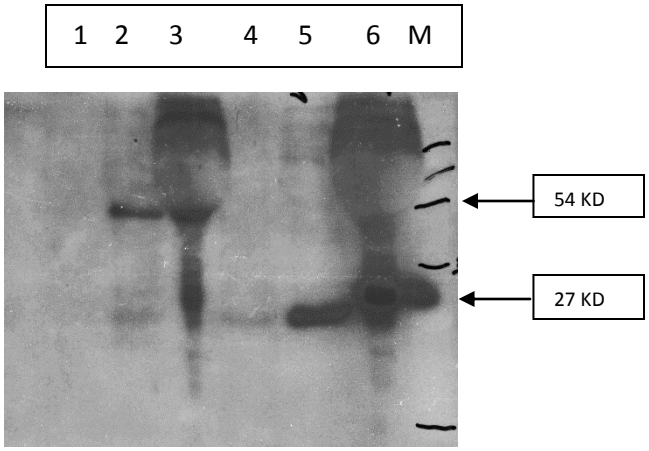
The resulting immunoblot blot indicates the expression of the Takeout-GST protein the recombinant experimental virus transfected cell cultures (Figure 37). The control virus infected culture shows expression of GST protein. The *takeout-GST* expression (experimental) and the *GST* expression (control) were seen in both the cell fraction and the supernatant fraction indicating only a part of the expressed protein was secreted to the medium. The Takeout-GST protein has the expected size of 53KD (Takeout (27KD) + GST (26KD)) size protein. Unfortunately, serum proteins have a similar size. This leads to the distortion of bands in this region on Western blots, and obscures Takeout protein on Commassie-stained gels.

(A)



1. GST (0.1 dilution)
2. GST (0.01 dilution)
3. GST (0.001 dilution)
4. Takeout-GST (0.1 dilution)
5. Takeout-GST (0.01 dilution)
6. Takeout-GST (0.001 dilution)

(B)



1. Takeout-GST (0.001 dilution)
2. Takeout-GST (0.01 dilution)
3. Takeout-GST (0.1 dilution)
4. GST (0.001 dilution)
5. GST (0.01 dilution)
6. GST (0.1 dilution)

Figure 37: Takeout-GST expression from BaculoGold-*takeout-GST* infected and GST expression from BaculoGold-*GST* infected Sf9 cultures. The above obtained Takeout-GST and GST proteins were subjected to serial dilutions of 0.1, 0.01 and 0.001 and analyzed using immunoblotting. Protein bands are visible in samples from the cell pellets (A) and the medium (B). Therefore, only a portion of the protein appears to be secreted.

The above culture medium samples of undiluted and 0.1 dilutions of Takeout-GST fusion protein and GST control protein were purified using GST purification. 1ml of each culture medium was concentrated and purified using GST-purification mini columns following the supplier's protocol. The elution step of the purification was repeated twice according to the protocol. The three elution samples were pooled and concentrated using Millipore concentrators. The samples were analyzed using immunoblotting with the α-GST antibody. 10µl of the undiluted and 0.1 diluted elution samples of Takeout-GST and GST protein were analyzed. Samples were also analyzed using Coomassie staining.

The immunoblot indicates clear bands for both the GST and the Takeout-GST proteins at undiluted and 0.1 dilution (Figure 38). The GST protein band is positioned at 26KD and the Takeout-GST band is positioned at 53KD. The amount of GST protein obtained is higher than the amount of Takeout-GST, probably due to the Takeout-GST protein being a fusion protein and therefore the expression and/or secretion of the protein being reduced.

These results show the presence of both proteins as secreted proteins and the ability to purify the proteins using GST purification. When the Takeout-GST and GST proteins were analyzed by PAGE and coomassie staining, the quantity of protein obtained was minimal and therefore, insufficient for binding studies.

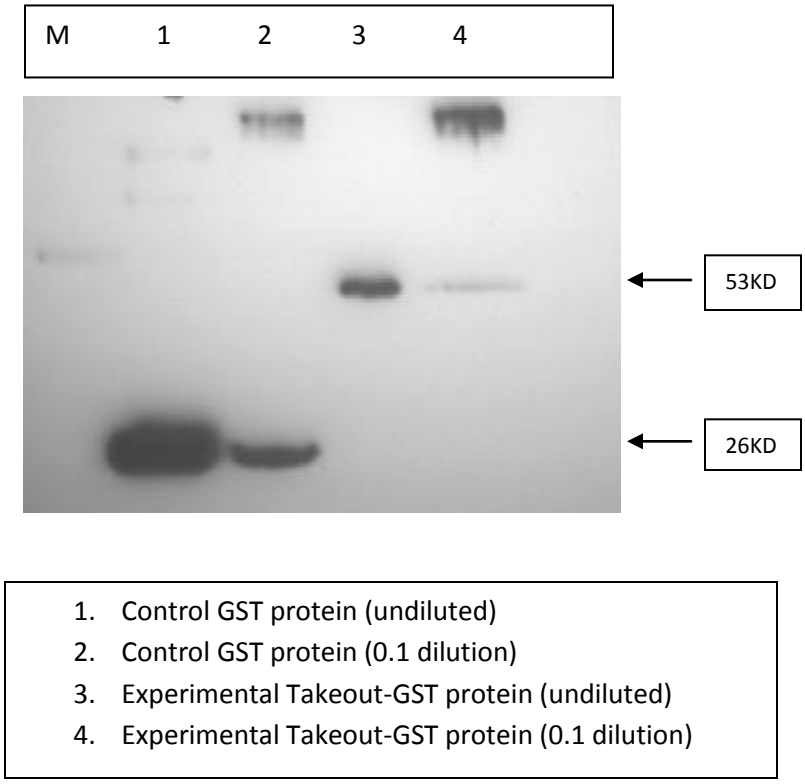


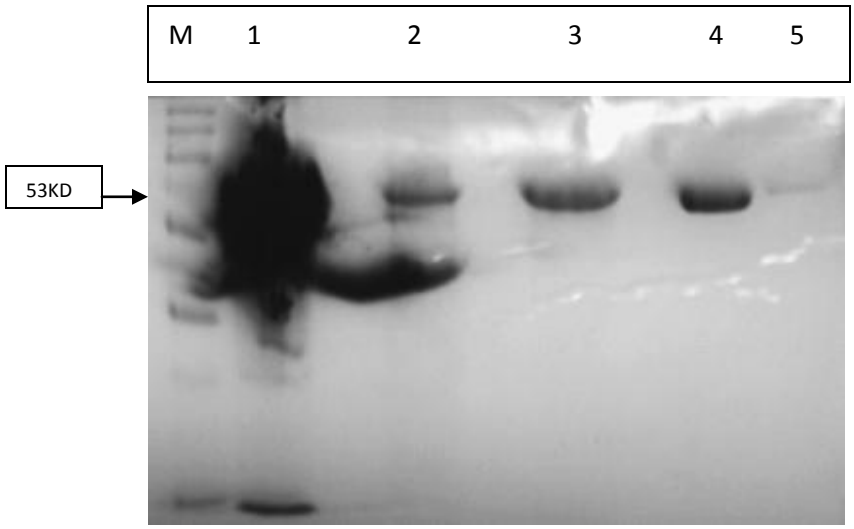
Figure 38: GST purification of Takeout-GST and GST proteins from Sf9 culture medium. The respective proteins were purified using Pierce Glutathione spin columns. The elution fraction was concentrated and analyzed using protein gels and coomassie staining for the presence of the GST protein expressed by the BaculoGold-*GST* virus and the Takeout-GST protein expressed by the BaculoGold-*takeout-GST* virus. The blot indicates the purification of Takeout-GST and GST proteins.

4.1.4. Characterization of protein expression in different Sf9 cell lines

In order to examine whether different cell lines would increase the yield of expressed protein, we collaborated with the company Allele Biotechnology Inc. They re-amplified our virus stock to create a high-titer stock and used it to infect three different types of Sf9 cell lines: Sf9, Sf9 Super, and Tni. Sf9 cells require serum, the Sf9 Super and Tni grow in serum-free medium. The protein was expressed in 30ml of culture which were sent to us for analysis.

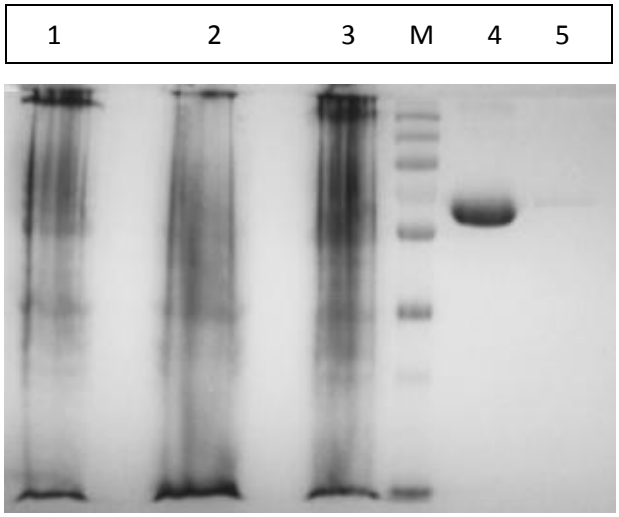
The culture media and cell pellets of the above cultures were analyzed for the presence and quantification of the Takeout-GST fusion protein. The culture media were prepared by concentrating 1ml samples of the cultures, mixing with Laemmli buffer, boiling and cooling. Approximately 1/10 (10%) of the 1 ml culture was analyzed. The cell pellet was analyzed by mixing with Lammli buffer, boiling and cooling. 100µl of total cell preparation (equivalent to 1ml of culture) which was concentrated to 10µl was analyzed. Culture media and cell pellets were analyzed using SDS PAGE and coomassie staining.

(A)



1. Sf9 medium
2. Sf9 super cell medium
3. Tni medium
4. BSA std. undiluted (14.5µg)
5. BSA std. 0.1 dilution (1.45µg)

(B)



1. Sf9 medium
2. Sf9 super cell medium
3. Tni medium
4. BSA std. undiluted (14.5 µg)
5. BSA std. 0.1 dilution (1.45 µg)

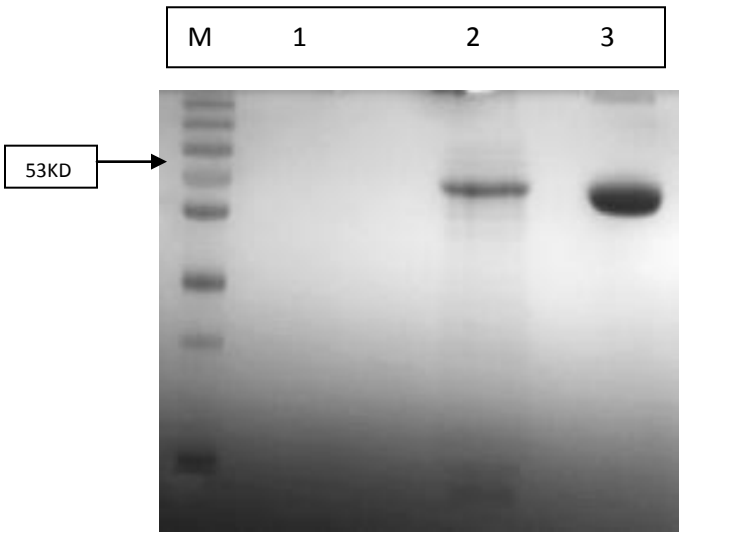
Figure 39: Coomassie analysis of Takeout-GST fusion protein expression from Sf9, Sf9 super cell and Tni cells. The cell fraction and supernatants were analyzed in separate protein gels. (A) Culture media of the three cultures were concentrated and 10% of 1ml culture equivalent was run in the gel. The presence of the Takeout-GST fusion protein is apparent in all three cultures, although since the Sf9 cell line grows in FCS (fetal calf serum) -containing medium, a large amount of serum protein that have a similar weight as Takeout-GST obscures the expressed protein. (B) The total cell pellet equivalent of 1ml of culture of the three culture media was run on the gel. The absence of the Takeout-GST protein in the cell pellets suggests the successful secretion of the protein from the cells into the culture medium.

The protein gels indicate the successful expression of the Takeout-GST protein in all three cell lines and that that the fusion protein is secreted into the culture medium (Figure 39). The absence of the protein in large quantities in our previous attempts is likely due to a low viral titer that was used for transfection and expression.

In comparison to the BSA standard, the amount of protein present in the Tni culture was estimated to be 14.5µg, whereas the amount present in Sf9 super cell medium appears to be approximately 10µg. The quantity of protein present in the Sf9 culture sample cannot be quantified, due to the presence of serum. We concluded from this analysis that a 30ml culture of Tni yields approximately 435µg of protein, while a 30ml culture of Sf9 super cell culture yields about 300µg of protein.

4.1.5. GST purification of the Takeout-GST fusion protein from Sf9 super cell medium

The Takeout-GST fusion protein in Sf9 super cell medium was subjected to GST purification. 10ml of culture medium was used for GST purification by the column purification method. The final purified eluted protein fraction was dialyzed against 50mM Tris-Cl (pH 8). The purified protein as well as the original un-purified protein was also concentrated. The purified and un-purified samples were analyzed by SDS PAGE and Coomassie staining. A BSA protein standard was also run alongside the protein samples.



1. GST purified protein/ Elution fraction
2. Un-purified original Sf9 super cell medium
3. BSA Std. undiluted (14.5µg)

Figure 40: GST purification of the Takeout-GST fusion protein from Sf9 super cell medium. The Sf9 super cell culture medium was subjected to GST column purification. The resulting purified protein in the elution fraction was analyzed by SDS PAGE and Coomassie staining. The stained gel shows the absence of the protein in the purified elution fraction.

The protein gel shows no Takeout-GST fusion protein in the elution fraction, in contrast to its presence in the original un-purified culture medium lane (Figure 40). It therefore appeared that the GST purification procedure used was unsuccessful. This could be due to the protein not binding the Glutathione agarose column and/or the protein not eluting from the column at the elution step.

4.1.5.1. Optimization of GST column purification

The previous experiment was unsuccessful in purifying the Takeout-GST fusion protein. While protein was present in the medium, it could not be bound to the column and was present in the flow-through.

A possible reason could be that the growth medium of the cells interferes with binding/elution, since concentrated unpurified medium was applied to the columns. I next performed a series of experiments. The following experiments were performed:

- dialyse against 1x PBS buffer prior to purification
- Batch purification of Takeout-GST
- precipitation of the protein by Ammonium Sulphate($(NH_4)_2SO_4$), followed by dialysis and column purification

A summary of results from these experiments is shown in Figure 41. Collectively, none of these modifications led to significant purification of the Takeout-GST fusion protein.

Dialysis against 1X PBS- The Sf9 supercell culture medium was dialyzed against 1X PBS buffer, GST purified, concentrated, and analyzed by Coomassie staining. The elution fraction does not indicate the presence of the protein. (Figure 41 A)

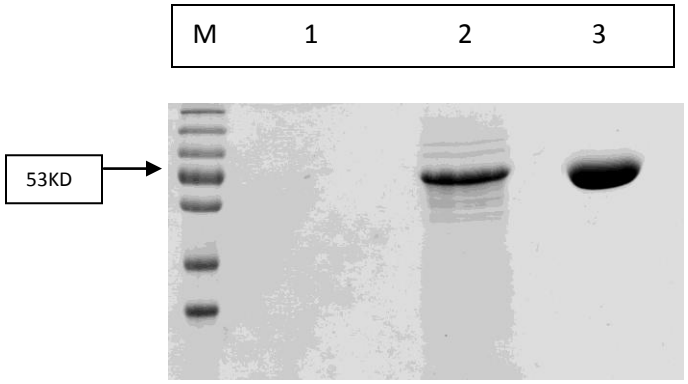
GST purification using Pierce Glutathione Mini-columns- When all three media, Sf9, Sf9 super cell, and Tni were GST purified using mini-columns, only Sf9 sample shows elution of a small amount of Takeout-GST protein(Figure 41 B). When the GST purification of all three culture media was analyzed by immunoblotting, a minimal, insignificant amount of protein was present in the elution fractions of all three culture media.

GST purification using the BD BioSciences batch purification method- The batch purification method was applied to Tni and Sf9 culture media. The protein bound glutathione beads were incubated with the Elution buffer for 3hrs and overnight in two separate experiments. Takeout-GST was eluted from the Tni medium after 3 hrs incubation of buffer with beads although the amount of protein purified was very insignificant. The Sf9 protein elution is not apparent due to the large amount of serum in the culture medium (Figure 41 C).

Ammonium Sulphate$(NH_4)_2SO_4$) precipitation and GST-purification of Takeout-GST- Takeout-GST protein in Tni medium was purified using Ammonium suphate precipitation and GST purified. The protein was not visible in the elution fraction when analyzed by Coomassie staining. Subsequently, the precipitated protein was subjected to GST purification at different pH values (6.9 and 9.0). The protein was not present in the elution fraction in both instances (Figure 41 D).

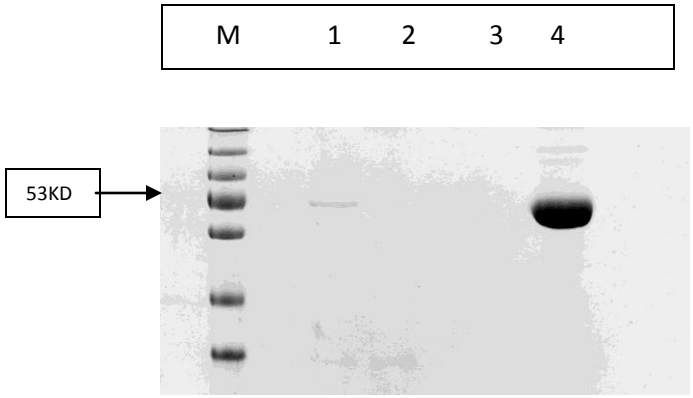
It is apparent, by the Coomassie gels in the above experiments that the Takeout-GST fusion protein is always present in the flow through of the purification experiment, indicating that the protein does not bind the Glutathione column.

(A)



1. Elution fraction of GST- purification
2. Flow through fraction of GST purification
3. BSA Std. undiluted (14.5µg)

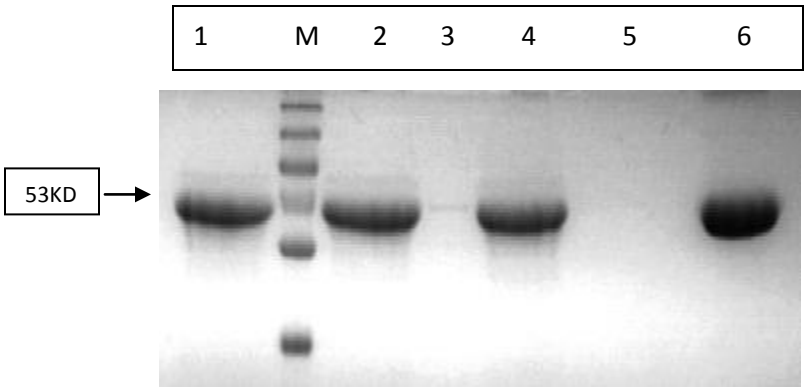
(B)



1. Sf9 medium
2. Sf9 super cell medium
3. Tni medium
4. BSA Std. undiluted (14.5µg)

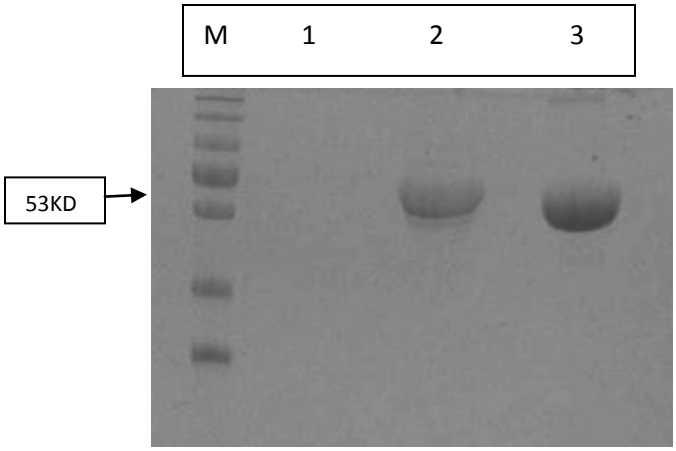
Figure 41 (A and B): Optimization of GST-column purification. The various attempts used to optimize Takeout-GST purification by GST purification method. (A) GST purification of Takeout-GST fusion protein after dialysis into 1X PBS buffer. (B) GST purification of Takeout-GST fusion protein using mini columns. The attempts to purify the protein by GST purification did not yield sufficient protein to be detected by coomassie staining.

(C)



1. Tni culture medium
2. Flow through after 3 hours incubation
3. Elution after 3 hours incubation
4. Flow through after overnight incubation
5. Elution after overnight incubation
6. BSA std. undiluted (14.5µg)

(D)



1. Elution of Takeout-GST
2. Flow through
3. BSA protein std. undiluted

Figure 41 (C and D): Optimization of GST-column purification. The various attempts used to optimize Takeout-GST purification by GST purification method. (C) GST purification of the Takeout-GST fusion protein from Tni medium using batch purification. (D) Ammonium Sulphate ($(NH_4)_2SO_4$) purification of Takeout-GST. The attempts to purify the protein by GST purification did not yield sufficient protein to be detected by coomassie staining.

Unfortunately, these expression test samples by the company did not include a GST only control sample. We learned later, when the company repeated the experiments at larger scale for protein purification, that the prominently expressed protein at 53KD was not Takeout-GST, but an unspecific virus protein. We were not able to recognize this in our initial expression experiments that included this control, because they were done in serum-containing media, and abundant serum-proteins of about the same size obscured the other proteins.

In order to obtain a larger amount of purified protein than we were able to get from smaller culture, we collaborated with Alelle Biotechnology Inc. They used the high-titer virus stock to infect 800 ml of cells and subsequently purified the fusion protein from the supernatant [Appendix 3]. The fusion protein was then delivered to us.

4.2. Enzymatic processing/ cleavage of Takeout-GST fusion protein

The Takeout protein was expressed *in vitro* as a Takeout-GST fusion protein to facilitate purification. However, for our binding studies the Takeout protein should be in its native form, detached from the GST tag in order to be used for JH^{H3} – Takeout-binding assays. Therefore, the fusion protein has to be cleaved to release the GST tag. The Thrombin cleavage site placed between Takeout and GST in the fusion protein facilitates this cleavage by acting as a recognition and cleavage site for the enzyme Thrombin.

The amount of Takeout-GST fusion protein processed for the binding reaction was 150µg. The protein was cleaved using the Thrombin enzyme solution. The protein was dialyzed overnight into cleaving buffer (50mM Tris-HCL (pH 8), 150mM NaCl, 2.5mM $CaCl_2$, 0.1% β-mercaptoethanol) and then incubated with the enzyme solution for 14 hours (0.49µl. 0.62U) The reaction mix was subsequently subjected to GST purification to purify the cleaved from the un-cleaved protein. The flow through contains the purified cleaved protein.

Immunoblotting and Coomassie staining were performed to verify the purification of the Takeout protein and for quantification. Samples of the cleaved ([2.1µg] 3% of fraction) and original fusion protein (0.025µg) were subjected to PAGE and immunoblotting using affinity purified rabbit raised α-Takeout primary and HRP-α-rabbit secondary antibodies. Samples of the above cleaved ([6.7 µg] 10% of fraction) and original fusion protein (5µg) along with 10µl of a BSA protein std. (1.45µg/µl) and 10µl of 0.1 dilution of the protein std were also subjected to PAGE and Coomassie staining.

The α-Takeout immunoblot (Figure 42 A) indicates the Takeout-GST fusion protein (53KD) as a clear band although with a small amount of degradation appearing as a faint band (27KD), and the processed and purified Takeout protein as a 27KD band. The processing and purification of Takeout-GST appears to be partial as un-cleaved and un-processed Takeout-GST (53KD) is also present in the cleaved and purified protein reaction. The cleaved reaction therefore, includes the pure Takeout protein (27KD), cleaved off GST protein (26KD) and the un-cleaved Takeout-GST protein (53KD).

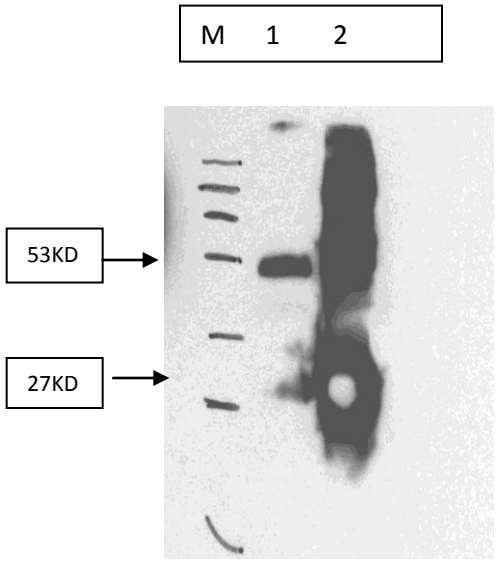
The Coomassie staining indicates a clear band of the un-cleaved Takeout-GST fusion protein (53KD) along with a faint band of degradation at 27KD and the processed and purified Takeout

protein as a 27KD band (Figure 42 B). The un-processed Takeout-GST (53KD) band that appeared at the immunoblot is not seen in Coomassie staining, probably due to reduced sensitivity of the Coomassie staining. This indicates that the amount of un-cleaved protein is considerably less compared to the processed pure Takeout protein. Additionally, the cleaved Takeout (27KD) band overlaps with the GST protein (26KD) band. Therefore, the quantity of Takeout protein is half of the quantity displayed in the band at the Coomassie gel. According to the protein standard bands, the quantity of Takeout protein obtained by the processing and displayed in the gel is approximately 1.08µg.

Therefore, the total amount of Takeout protein obtained is 86.4µg.

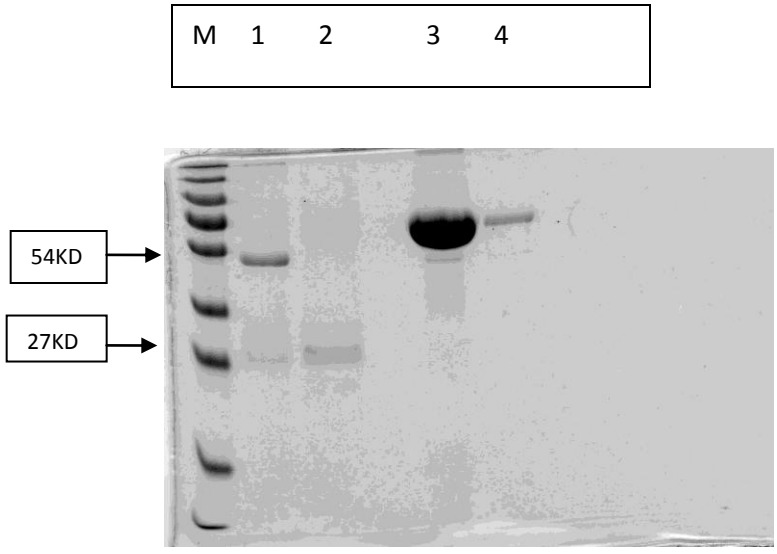
The final cleaved protein was concentrated and used for binding assay with JH^{H3}.

(A)



1. Original Takeout-GST
2. Processed Takeout-GST indicating Takeout protein

(B)



1. Original Takeout-GST
2. Processed Takeout-GST indicating Takeout protein
3. Protein std. BSA (14.5 µg)
4. Protein std BSA 0.1 dilution (1.45 µg)

Figure 42: Takeout-GST cleaved into Takeout and GST proteins by Thrombin. The Takeout-GST fusion protein was cleaved using the Thrombin enzyme. (A) The un-cleaved original protein and cleaved, purified protein were subjected to PAGE and immunoblotting. The blot was incubated with an affinity purified α-Takeout anti-body and HRP-α- rabbit antibodies. The figure indicates the Takeout-GST fusion protein (53KD) and the processed and purified Takeout protein (27KD). The enzymatic cleavage and purification appears to be partial as the blot also displays a band corresponding to un-cleaved GST fusion protein (53KD) along with the pure Takeout protein (27KD) band. (B) The un-cleaved original protein and cleaved, purified protein were subjected to PAGE and Coomassie staining along with protein standards. The original un-cleaved protein appears as a single clear band at 53KD. A minimal amount of degradation is also apparent. The processed and GST purified protein appears as a single clear band at the 27KD position.

4.3. Binding between JH^{H3} (tritium labeled Juvenile hormone) and Takeout- equilibrium dialysis

The processed Takeout protein was used for the equilibrium dialysis binding reaction with JH^{H3} (tritium- labeled Juvenile hormone). The processed, GST-purified protein was concentrated as mentioned above. 64.8µg of purified Takeout protein was used for the binding assay. The protein was placed in dialysis tubing and dialyzed overnight into Juvenile hormone-binding buffer (50 mM Tris, 100 mM KCl pH=7.0). The Juvenile hormone was prepared by adding 1500 dpm of JH^{H3} per 125ml of binding buffer in a beaker. The buffer containing the JH^{H3} was evaporated using vacuum. The Takeout protein, now dialyzed into binding buffer was placed in dialysis tubing and placed in the binding buffer at 4°C for 4 days on a stirrer. Samples of 200µl each of the protein sample and binding buffer were analyzed for the presence of Juvenile hormone, using a scintillation counter. A higher amount of dissociation counts at the protein sample compared to the buffer sample indicates specific binding between the protein and the hormone.

The scintillation counter readings were taken for two experiments.

The first experiment was done in two replicates. Two samples of protein were subjected to the equilibrium dialysis experiment. The scintillation counter readings were obtained for the protein sample inside the dialysis tubing and the outside buffer along with the fresh dialysis buffer as control. The readings for JH^{H3} are as follows-

Replicate	Control	Buffer	Protein sample
1	8.00	110	105
2	8.00	101	99

According to the above readings, the protein inside the dialysis tubing and the equilibration buffer containing JH^{H3} did reach equilibrium. However, the readings inside the dialysis bag do not show an increase over the readings outside the dialysis bag. Therefore, the experiment indicates the absence of specific binding between Takeout and JH^{H3}.

The above experiment was repeated with a fresh sample of cleaved Takeout protein (64.8 µg). The experiment was done for a single replicate because we did not have more protein.

The scintillation counts obtained are as follows-

Replicate	Control	Buffer	Protein sample
1	6	57	48

Again, the above readings do not indicate an increase in Juvenile hormone levels inside the dialysis bag when compared to outside the bag. Therefore, the experiment indicates the absence of specific binding between Takeout and JH^{H3}.

In summary, these equilibrium dialysis experiments do not show binding of Juvenile hormone to the Takeout protein.

CHAPTER V

DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER 5: DISCUSSION AND FUTURE PERSPECTIVES

5.1 Juvenile hormone plays a role in male courtship behavior in *Drosophila melanogaster*

This project explores if Juvenile hormone plays a role in male courtship behavior in *Drosophila melanogaster*. Our findings indicate that disruption of Juvenile hormone synthesis by targeted reduction of *JHAMT* in flies reduces courtship. *JHAMT* expression was reduced by RNAi and by genetic ablation of the site of Juvenile hormone synthesis, the Corpora allata. Expression of *JHAMT*-RNAi resulted in a mutant phenotype when RNAi was expressed throughout development (Figure 20) or conditionally in adults using the heat-inducible *hsp70-GAL4* promoter (Figure 24). In all of these experimental conditions, the defect could be rescued by the application of the Juvenile hormone analog, Methoprene in mature males shortly before performing the courtship assay (Figures 21 and 25). This indicates that *JHAMT*-RNAi expression resulted in lower Juvenile hormone levels. Our results also indicate that the hormone is required physiologically in adult mature males for normal courtship, and that the observed effect is not due to a developmental requirement of Juvenile hormone for courtship.

The finding of this study of a requirement for Juvenile hormone in courtship behavior is a novel finding. Juvenile hormone mainly functions in the development of insects. It is known for its crucial role in maintaining the larval stage in insects between molts until a desired critical weight is reached (Hartfelder, 2000; Nijhout and Williams, 1974a, b). When the critical weight is reached, the Juvenile hormone levels decrease, facilitating molting into the next larval stage.

The role of Juvenile hormone during development is very well studied. In contrast, there are few findings of functions for Juvenile hormone in adults. Most of its adult described functions are in

reproduction, a majority of them in females. Juvenile hormone was shown to be involved in oocyte maturation (Dubrovsky et al., 2002; Soller et al., 1999). The hormone functions in the regulation of yolk protein genes and thereby the synthesis of yolk proteins (Bownes M, 1994). Soller and colleagues (Soller et al., 1999) have shown that mating and sex peptide injection and the application of the Juvenile hormone analog, Methoprene stimulate oocyte development through a control point between development stages 9 and 10. Both Juvenile hormone and 20-hydroxyecdysone (20E) have been implicated in regulating vitellogenesis (yolk protein production and uptake by the growing oocyte) (Bownes M., 1986, Riddiford L.M., 1993). In female flies, Juvenile hormone was shown to regulate oocyte maturation and reproductive activity (Riddiford, L.M., 1993; Wyatt et al., 1996; Bownes, M., 1992; Bownes, M., 1989; Handler and Postlethwait, 1977; Jowett and Postlethwait, 1980; Wilson et al., 1983). A reduction in Juvenile hormone leads to a halt in development of oocytes past the 9th stage (Wilson, T.G., 1982). Oocyte development is therefore a good indicator of Juvenile hormone levels that I have used in my studies. I have shown that the expression of *JHAMT*-RNAi resulted in smaller ovaries, consistent with a reduction in Juvenile hormone (Figure 29).

Juvenile hormone has been shown to have an effect on several post-mating behaviors in females. In *Blatella germanica* (cockroach), production, and release of sex pheromones by the female is regulated by Juvenile hormone (Schal et al., 1997). However, a further increase in reproductive activity was observed in the above study after the transfer of seminal fluid into the female. Previous research indicates the activation of Juvenile hormone synthesis (JHB3) by sex peptide in seminal fluid which is transferred to the female by the male during mating (Fan et al., 1999; Moshitzky et al., 1996). After mating, the sex peptide in the seminal fluid stimulates Juvenile hormone synthesis in the Corpora allata and consequently stimulates egg deposition

while reducing female receptivity (Kubli, 2003). Juvenile hormone was also shown to promote protein synthesis in the accessory glands of males (Riddiford L.M., 1993; Gillott, 2003; Wilson et al., 2003).

In our study, we performed a reduction of Juvenile hormone in flies to explore its effect on mating behavior. Several previous studies have used a less direct approach to study the role of Juvenile hormone levels. One of them used *ap* mutants. Many *ap* mutations decrease the rate of Juvenile hormone production in the Corpora allata (Altaratz et al., 1991). The *ap* mutation leads to several phenotypes in the adult (Butterworth and King, 1965), among them non-vitellogenic oocyte development and failure of larval fat body histolysis. These defects are likely due to Juvenile hormone deficiency as they can be rescued by the application of the synthetic hormone (Gavin and Williamson, 1976; Postlethwait and Jones, 1978; Postlethwait and Weiser, 1973). *ap* mutant (ap^4) females were shown to have a reduced amount of Juvenile hormone synthesis (Bodenstein, 1947; Handler and Postlethwait, 1977). These observations suggest that the *ap* gene product is involved in Juvenile hormone synthesis or secretion. However, the mechanisms for this function are not known yet. Furthermore, studies have shown a defect in male sexual maturation in *ap* mutant *Drosophila melanogaster* that might be related to the delay in fat body maturation observed in *ap* mutants (Ringo et al., 1992; Tompkins, 1990). These studies suggested a relationship between Juvenile hormone and courtship behavior in *Drosophila malanogaster*, although this had not been directly shown prior to our studies. A study in Caribbean fruit flies(*Anastrepha suspense*) indicates that Juvenile hormone is required for sexual signaling and reproductive maturity (Teal et al., 2000).

To further verify the above findings I conditionally ablated the Corpora allata using cell death constructs. The *UAS-DTI* construct, when driven by *JHAMT-GAL4* in the Corpora allata conditionally using the *Gal80^{ts}* system, resulted in a reduction of courtship behavior (Figure 28). This ablation of the site of hormone synthesis is an indirect way to reduce hormone levels in flies. It was done conditionally to facilitate proper growth and development during the larval stages. The resulting reduction in courtship index was similar to that obtained by expression of of RNAi against *JHAMT* (Figures 20, 24). This could indicate that the reductions we observed reflect the extent to which Juvenile hormone contributes to the regulation of courtship. However, it is possible that only a partial ablation of the organ was achieved. To examine the extent to which cell ablation has acted in the organ, the flies need to be sectioned and examined by immunohistochemistry using an α-JHAMT antibody.

Previous research has shown that Juvenile hormone acts through the *Met* transcription factor (Abdou et al., 2011; Baumann et al., 2010; Konopova and Jindra, 2007; Miura et al., 2005; Wilson and Fabian, 1986). *Met^{27}* mutants were found to display reduced courtship, indicating a role for *Met* and Juvenile hormone in courtship, although the courtship defect could not entirely be rescued by a rescue construct in that study (Wilson et al., 2003). This is in agreement with our findings.

Methoprene has a high structural similarity to Juvenile hormone. Methoprene, as well as other compounds such as Pyriproxifen, Phenoxycarb etc have been known to be Juvenile hormone analogs (Jindra et al., 2013; Riddiford and Ashburner, 1991) and have been previously used to mimic Juvenile hormone action (Chamseddin et al., 2012; Dubrovsky et al., 2002; Miura et al., 2005; Riddiford et al., 1991; Soller et al., 1999; Zhou and Riddiford, 2008). Methoprene is more

stable than Juvenile hormone and is therefore often used in place of the hormone, as I did in my studies. However, prior to the current study, Methoprene has not been used to rescue a behavior mutant phenotype in insects.

When I used Methoprene to rescue the courtship phenotype caused by reduced Juvenile hormone levels, the courtship index of the experimental genotypes increased back to normal while those of the two control genotypes very consistently decreased (Figures 21 and 25), although this decrease did not reach significant statistical levels. As discussed before, these rescue experiments showed that normal Juvenile hormone levels are required for normal courtship in flies. In addition, the observed trend for reduction of courtship index in control genotypes upon treatment with Methoprene suggests that above normal levels of Juvenile hormone also have an effect on the flies' courtship and suggests that an optimal level of Juvenile hormone is required for normal courtship. Previous research (Adam et al., 2003) has shown that an above normal level of a Juvenile hormone analog, Pyriproxifen causes male genitalia rotation in *Drosophila* larvae. Similarly, the Juvenile hormone analogs Methoprene and Pyriproxifen, when topically applied at low doses to white pre-pupae, produce genitalia rotation (Riddiford and Ashburner, 1991).

We were able to rescue the courtship reduction that was caused by RNAi against *JHAMT* by application of the Juvenile hormone analog, Methoprene. Furthermore, we have observed ovary defects in females of this genotype. Together this strongly indicates that our RNAi treatment against *JHAMT* RNA resulted in a reduction of *JHAMT* RNA leading to reduced Juvenile hormone levels. In order to examine this molecularly, we performed qPCR analysis of *JHAMT* RNA levels in the mutants. qPCR was performed on flies used for RNAi mediated disruption of Juvenile

hormone synthesis and flies with genetic ablation of the Corpora allata. The qPCR results did not confirm a reduction of *JHAMT* mRNA (Figures 31, 32, and 33). This would indicate that the expression of *JHAMT*-RNAi did not disrupt the enzyme and that disruption of Juvenile hormone biosynthesis did not occur. This is in contrast to our finding of a courtship mutant phenotype that was rescued upon treatment with Methoprene, indicating that a reduction in Juvenile hormone levels led to this phenotype. Several control experiments could be done to examine this discrepancy. First, expression levels of the RNAi construct itself should be verified. The expression levels of the RNAi could be tested by qPCR. In order to obtain an optimal knockdown the *JHAMT* mRNA levels should be approximately equal to the *JHAMT* -RNAi levels. If the RNAi expression is insufficient, the mRNA knockdown may not be efficient.

Second, the validity of the qPCR reactions could be examined. While the TaqMan probe used is the only one available, qPCR using different primers could be performed using the SYBR-Green system. Furthermore, expression of an unrelated RNAi construct (such as one directed against GFP, for example) could be used to verify that it does not disrupt courtship.

If these control experiments agree with our results, several possible explanations could account for our findings. For example, the reduction in Juvenile hormone levels obtained through our manipulations might be minimal. Rescue of the effect by application of Methoprene indicates that small reductions in Juvenile hormone levels are capable of affecting courtship. A minimal decrease in Juvenile hormone levels by RNAi is consistent with our finding that even RNAi carried out throughout development did not lead to developmental disruption, and that the flies had normal activity levels in comparison to controls (Figure 20). The levels of Juvenile hormone in flies during development are much higher than in adults (Dai and Gilbert, 1991). Therefore,

the RNAi probably did not decrease Juvenile hormone levels in larvae sufficiently to affect development.

An alternative explanation for our inability to see a reduction in *JHAMT* RNAi by qPCR would be that *JHAMT* is expressed at fairly high levels in other places than the Corpora allata, so that a reduction only in this organ would not be recognizable in the qPCR assay. However, *JHAMT* was shown to be specifically expressed in the Corpora allata, when examined by antibody staining (Niwa et al., 2008); although, the authors have not examined whole body staining, and it is possible that *JHAMT* expression may exist in other locations of the body. It is well known that the relevant organ of Juvenile hormone synthesis in insects is the Corpora allata (Judy et al., 1973; Roller and Dahm, 1970) for the Juvenile hormone functions that have been examined. The *JHAMT-GAL4* does show some expression in other tissues (Figure 19 B), but we do not know to what extent this reflects endogenous *JHAMT* expression. The RNA expression data in Flybase (http://flybase.org/reports/FBgn0028841.html) indicate presence of *JHAMT* RNA in heads and brains, but also in the male accessory glands at fairly high levels. The reason for the expression of the *JHAMT* gene in these tissues is not clear. But the relatively high levels of expression in these tissues might be able to explain our qPCR results. To address this question, examination of the expression of the *JHAMT* gene should be performed in the fly by whole body *in situ* hybridization. The expression levels of experimental *JHAMT* knockdown and control genotypes could be examined for reduction in expression in all areas where *JHAMT* expression is expected. Our qPCR experiments also indicate a fairly large variation in basal *JHAMT* RNA levels in different genetic backgrounds, for example in the heat shock experimental and controls; the control genotypes appear to have different levels of *JHAMT* mRNA, the reason for this is not clear. This

consequently complicates the analysis of the effect of RNAi on the *JHAMT* transcript. To address this issue, out crossed strains would need to be examined.

The qPCR experiment performed on flies of the *hsp70-GAL4* induced RNAi experiment indicates a universal reduction in *JHAMT* mRNA levels in controls and experimental genotype upon induction (Figure 32). However, we do not observe a reduction in courtship behavior in the heat-induced controls upon induction (Figure 24). Consequently, the behavioral phenotype observed by conditional *JHAMT* RNAi in adults contradicts the qPCR observation on levels of *JHAMT*. The consistent reduction in mRNA levels observed in qPCR would suggest that the controls as well as the experimental should show a courtship mutant phenotype. However, the controls show a normal courtship phenotype (Figure 24). A possible explanation could be that reduction in hormone levels that could occur by *JHAMT* knockdown could affect a physiological pathway at the initial stages of induction. This could consequently cause a reduction in courtship index in the flies of only the experimental genotype, where *JHAMT* knockdown is performed. However, the mRNA levels in flies are examined at 4 hours after induction, when the RNA is examined. It is well known that the heat shock and stress response results in sequestration and reduction of mRNAs (Sorensen et al., 2005). This question could be addressed by performing a time course, examining *JHAMT* RNA levels at different time points following heat shock.

Another possibility could be that the courtship mutant phenotype could be a response to the activation of the RNAi machinery in the body, and not due to a reduction in *JHAMT* levels (although it would be unclear why this effect would be rescued by Methoprene). This could be examined using a scrambled RNAi construct. However, scrambled RNAi controls are not usually

used in the *Drosophila* system, as it is a complex organism and scrambled RNAs are more likely to lead to un-specific effects. Therefore, an RNAi construct directed against GFP could be used.

It is important to show the reduction of Juvenile hormone titers in these experiments. The *JHAMT* knockdown could reduce *JHAMT* levels, although not clearly apparent in the qPCR experiments. However, *JHAMT* levels are not directly indicative of Juvenile hormone levels in the hemolymph. JHAMT is the enzyme that is involved in the Juvenile hormone biosynthesis pathway at the conversion of Farnesoic acid to Juvenile hormone. As Juvenile hormone is not a direct product of the *JHAMT* gene, a slight reduction of *JHAMT* may affect the Juvenile hormone levels in the fly significantly. The findings on ovary maturation under *JHAMT* RNAi conditions and the rescue experiments strongly support our interpretation of reduced Juvenile hormone levels in the mutants. To confirm the reduction of the hormone, its titer in the adult flies could be determined. Another method to determine an impact by *JHAMT* RNAi would be to perform a *JHAMT* activity assay as done by (Gruntenko et al., 2010).

We observed a comparable courtship reduction when *JHAMT* knockdown was carried out throughout development or when carried out conditionally in adults, through RNAi and Corpora allata cell ablation (Figures 20, 24, 27, and 28). This could indicate that this is the extent to which Juvenile hormone levels contribute to the regulation of courtship behavior, and that additional lowering of Juvenile hormone would not reduce the phenotype further. This interpretation would also be supported by another experiment I performed. In an attempt to investigate if the effect of Juvenile hormone on courtship behavior is dose dependent, we created *JHAMT-GAL4*; *Gal80^{ts}* flies carrying two *JHAMT-RNAi* copies (Figure 22, and 23). I did not observe a stronger effect than was observed with just one copy, although the *Gal80^{ts}* system

used for these experiments appears to be very weak and did not give a phenotype, in contrast to *hsp70-GAL4* conditional experiments. However, the reduction in Juvenile hormone titers would need to be examined in the above experiments to determine if the reduction is comparable. It is the only way to verify that the very similar courtship reduction observed in the different experiments is because of a threshold level of Juvenile hormone titer has been reached, independent of the actual reduction levels. Alternatively, the titer reduction in all of the above experiments could be equal, which could be the reason for a comparable reduction in courtship index.

JHAMT knockdown has been used in other previous research studies to reduce Juvenile hormone levels (although with different drivers than the one we created). These experiments have not shown consistent, robust results (Niwa et al., 2008). The above study, while using a different *UAS-JHAMT-RNAi* line from the one that we used, has not shown a knockdown of *JHAMT* mRNA in adults. While *JHAMT* has consistently been show to be a key enzyme for Juvenile hormone synthesis in other insects, it is formally possible that the enzyme does not play the same role in *Drosophila melanogaster*, and that another enzyme plays a more important similar role. Therefore, action of *JHAMT* may not be the most significant regulatory step in the synthesis pathway of Juvenile hormone. Furthermore, a reduction in Juvenile hormone levels may activate a positive feedback mechanism that would enhance *JHAMT* expression, thereby, preventing the complete RNAi effect in flies. This could be a reason for the insignificant reduction in *JHAMT* mRNA levels that I observed. Future experiments are required to address this question. Further experiments could attempt to reduce *JHAMT* levels by using RNAi machinery alongside a deficiency for *JHAMT*. However, the reduction in Juvenile hormone levels should be examined to verify an increase in hormone reduction.

5.2. How could Juvenile hormone affect courtship?

The possibility that Juvenile hormone plays a role in male courtship was hypothesized based on previous findings of similarity between JHBPs and Takeout (Dauwalder et al., 2002; Sarov-Blat et al., 2000; So et al., 2000). If the Takeout protein is a JHBP, it is possible that a mutation in *takeout* would act in the same genetic pathway as a mutation that reduces Juvenile hormone levels in the fly. Therefore, if Takeout and the hormone act in the same genetic pathway by binding to each other, then mutations in both would not be additive. If they act in separate pathways, they would show an additive effect on the behavior. However, since *JHAMT* RNAi is weak, it is likely that the reduction we induce in our *JHAMT* RNAi flies is not maximal. If Juvenile hormone levels were reduced in a *takeout* mutant background, the reduction in courtship index might be equal to the maximum level obtained when they are individually mutant. Therefore, this study set forth to examine if such a genetic interaction does exist.

Previous research has shown a courtship mutant phenotype in *takeout* homozygous mutants and its genetic interaction with a *fru* heterozygous mutant background to further reduce the courtship index (Dauwalder et al., 2002). The above study also demonstrates that *takeout* heterozygous mutants court normally. The present study aimed to investigate if reduced Juvenile hormone levels could genetically interact with a *takeout* heterozygous mutant background to produce a stronger reduction of the courtship index. To achieve this, the *JHAMT-GAL4* driver was used to drive *UAS-JHAMT-RNAi* in a *takeout* heterozygous mutant background. The disruption of Juvenile hormone synthesis was carried out throughout development to achieve a maximum level of knockdown. The results did not indicate a genetic interaction between reduced Juvenile hormone levels and a *takeout* heterozygous mutant background

(Figure 26). The observed courtship reduction was the same as the one observed with the *JHAMT* knockdown alone. This could be indicative of the two mutant conditions acting on the same genetic pathway. However, it is also possible that the amount of Takeout protein produced by a Takeout heterozygous mutant is equal to that of a wildtype. The amount of protein produced by heterozygous and homozygous mutants could be examined and compared to confirm this. As a follow up to this experiment, *JHAMT* knockdown can be attempted in a *takeout* homozygous mutant background. If under a homozygous mutant condition, which has been previously shown to have a courtship mutant phenotype, (Dauwalder et al., 2002), courtship levels do not further decrease, we would conclude Takeout may bind the hormone and act in the same genetic pathway.

Previous studies have proposed a model of Juvenile hormone binding to JHBPs in close proximity to the Corpora allata, and its release at the target cells. This model was based on two conformational states of the Juvenile Hormone Binding Proteins of *Bombyx mori* (*Bm*JHBP) of apo-JHBP and JH-bound JHBP. According to this model, the release of the hormone from JHBPs could occur at target cells based on a decrease in dielectric constant (Suzuki et al., 2011). However, the mechanism of recognition of target cells by Juvenile hormone or JHBPs has not been identified. Furthermore, the presence of membrane receptors or membrane transporters for Juvenile hormone has not been shown.

Juvenile hormone is a lipophilic sesquiterpene and therefore it is possible that the hormone enters the cell through the cell membrane passively. Therefore, it is suggested that an intracellular receptor may be present for the hormone. The Methoprene tolerant gene (*Met*) was discovered through a screen for *Drosophila* mutants resistant to Methoprene (Wilson and

Fabian, 1986). *Met* is a bHLH-PAS transcription factor (Ashok et al., 1998). A study by Miura and colleagues has shown binding of recombinant *Drosophila* Met to Juvenile hormone at physiological conditions. The study also demonstrates weak Juvenile hormone and Methoprene dependent transcriptional activation *in vitro* (Miura et al., 2005). The authors performed transient transfection assays using *Drosophila* S2 cells. They show that the yeast GAL4-DNA binding domain fused to *Met* protein activated a reporter gene when bound to Juvenile hormone or the Juvenile hormone analog (JHA), Methoprene. The activation by Methoprene was shown to be comparatively less than the activation by Juvenile hormone. When *Met* mutants were first characterized, it was somewhat surprising that *Met* mutants were viable, suggesting that it might not be the only receptor for hormone. Recent reports show that Met may functionally overlap with Gce, its paralog protein (Baumann et al., 2010) and that only the loss of both the genes confers lethality (Abdou et al., 2011). *Met* has been shown to function through *Kr-h1*(Minakuchi et al., 2008b). The gene *br*, is a zinc-finger transcription factor involved in Juvenile hormone action (Zhou and Riddiford, 2002). *br* is expressed predominantly during larval pupal transition (Emery et al., 1994; Huang et al., 2011), as its expression is inhibited by Juvenile hormone (Zhou et al., 1998; Zhou and Riddiford, 2002). Previous research has shown diminished expression of *Kr-h1* and enhanced expression of *Br* in Met^{27} and $Gce^{2.5K}$ double mutants, suggesting that under reduced levels of Juvenile hormone, the expression of *br* is enhanced (Abdou et al., 2011). Therefore, it is thought that *Met* and *Gce* act together to transduce Juvenile hormone signaling and that *br* acts downstream of the signaling pathway. These studies have been done during development. The signaling pathways that function downstream of Juvenile hormone in adult cells have not been studied as yet and may be different to the pathways in development.

Our study shows that Juvenile hormone has a role in male courtship behavior in flies. In order to affect the behavior it needs to affect the courtship neural circuits, either directly or indirectly. If Juvenile hormone is to affect the courtship neural circuits in the brain, it should pass through the blood brain barrier, either by itself or with the help of a transporter. Furthermore, once inside the brain, Juvenile hormone should interact with surface receptors of the neurons to initiate a signaling or transcription cascade that would activate them. Further research will be required to investigate the exact mechanism of interaction of Juvenile hormone with the courtship neural circuits.

5.3. *In vitro* expressed Takeout does not bind tritium-labeled Juvenile hormone (JH^{H3})

The above experiments indicate that disruption of the synthesis of Juvenile hormone affects courtship behavior. As previously mentioned, considering the sequence similarities in the N-terminal region of known JHBPs, it is possible that Takeout may act as a JHBP and act as a transporter for Juvenile hormone in the hemolymph of the fly. Takeout could function to transport Juvenile hormone to the courtship neural circuits or other target cells, thereby facilitating Juvenile hormone's effect on the courtship neurons. This could be a mechanism for the action for Juvenile hormone in courtship behavior. As an initial attempt to test this hypothesis, the binding between Takeout and Juvenile hormone was studied. Takeout was expressed *in vitro* as a Takeout-GST fusion protein using the Baculovirus expression system in insect cells. Insect cells were used for this purpose as it is a eukaryotic insect system as opposed to the *E.coli* system, thereby facilitating insect specific modification of the protein. The binding

between Takeout and JH^{H3} was tested using equilibrium dialysis experiments. Equilibrium dialysis has been used previously to study the binding between hemolymph JHBPs and radio-labeled Juvenile hormone of *Manduca sexta* (Park et al., 1993).

When equilibrium dialysis was performed between JH^{H3} and the pure (processed and GST-purified) Takeout, binding between them was not observed. There could be several reasons for this observation. It is possible that Takeout does not bind Juvenile hormone and thereby, it may not mediate the action of Juvenile hormone in courtship behavior in flies. Juvenile hormone may act to affect courtship behavior independently of Takeout. It is also possible that although the binding may occur *in vivo*, it may not occur *in vitro*. There may be factors in the hemolymph of the fly that facilitate the binding, and in their absence binding does not occur. It is also possible that the native Takeout produced in the fly has subtle differences in structure that facilitate its binding with Juvenile hormone, while the *in vitro* expressed protein does not. Previous research has used the native JHBP of *Manduca sexta* isolated from hemolymph to study binding to Juvenile hormone (Park et al., 1993).

Additionally, it may be possible that the equilibrium dialysis experiments performed to explore the binding may not be effective. As a control, the binding between Met and JH^{H3} could be examined in parallel by equilibrium dialysis.

The above results indicate that further experiments are required to examine potential binding between Juvenile hormone and Takeout.

Possible binding between Takeout and Juvenile hormone was hypothesized based on the similarity of the proteins, particularly the 100% conservation of the pair of N-terminal Cys residues (Dauwalder et al., 2002; Sarov-Blat et al., 2000; So et al., 2000). The only Takeout

structure determined so far is that of *Epiphyas postvittana* Takeout1 (*Ep*To1) (Hamiaux et al., 2009). *Epiphias postvitana* Takeout1 is a distant relative of *Drosophila melanogaster* Takeout, as determined by the phylogenetic analysis performed in our lab (N. Vanaphan and R. Zufall, unpublished results). *Epiphyas postvittana* Takeout was expressed in *E. coli* and crystallized. It was found co-crystallized with Ubiquinone. A surrogate Ubiquinone ligand was found deeply bound in the *Ep*To1 protein in a 45 Å long, purely hydrophobic tunnel. This was the first evidence of ligand binding by a Takeout protein (Hamiaux et al., 2009). Crystallographic studies that have been carried out on *Bm*JHBP idenitifed a relatively smaller cavity that Juvenile hormone binds to (Fujimoto et al., 2013; Suzuki et al., 2011). Given this difference, considering the similarity of *Epiphias postvittana* (*Ep*To1) and *Drosophila melanogaster* (*Dm*To1)Takeout proteins, this may be another indication that *Dm*Takeout may not bind Juvenile hormone, but another, as yet unknown ligand. On the other hand, *Dm*Takeout and *Ep*Takeout are distant relatives as discovered in our lab (un-published data), and therefore, their ligand-binding properties may vary. Future experiments need to address this question. A very recent publication by (Hamiaux et al., 2013) describes the crystal structure of the Baculovirus-produced *Epiphyas postvittana* Takeout protein (*Ep*To1). The observed structure is very similar to the one obtained with the *E. coli*-produced protein. However, in the Baculovirus system, the protein was found to co-crystallize with fatty acids.

The binding assay between Takeout and Juvenile hormone was a quest to identify a JHBP for *Drosophila melanogaster*, based on a hypothesis of Takeout being a JHBP. A JHBP has not been as yet identified for *Drosophila*. If Takeout is not a JHBP, it remains to be seen whether there are other hemolymph proteins that transport to Juvenile hormone.

5.4. Conclusion

In summary, this study has demonstrated a function for Juvenile hormone, a hormone that is largely regarded in insect development, in courtship behavior in *Drosophila melanogaster*. The study was principally based on the hypothesis that the Takeout, a male specifically expressed protein in the adult fly, acts as a JHBP in the hemolymph; as a transporter for Juvenile hormone. I have experimentally demonstrated a courtship function for Juvenile hormone in the adult male fly. Preliminary binding assays performed between Juvenile hormone and Takeout *in vitro* do not indicate binding between the hormone and the protein.

Further experiments are required to explore the mechanism of action of Juvenile hormone and its signaling pathways that function to affect this behavior. Additionally, further experiments on Takeout and Juvenile hormone binding are required to verify that Takeout is not a JHBP.

APPENDIX

Appendix 1

A. *JHAMT* upstream promoter sequence used for the construction of the *JHAMT-GAL4* driver

B. *JHAMT* gene sequence

C. pSC-B vector

D. pPTGAL vector

E. Alignment of the *JHAMT* upstream promoter region inside the pPTGAL vector using restriction sites

A. *JHAMT* upstream promoter sequence used for the construction of the *JHAMT-GAL4* driver (5043 bp)

5'-

tgcggtttaggggtgctatgacttgactttcagtctgtgcgggaaaaggtcatagcctgagataaggcttaatcgaaacttatgctatgtaaaaacttataaattaaaatagaaaataataaaggtttctcgc
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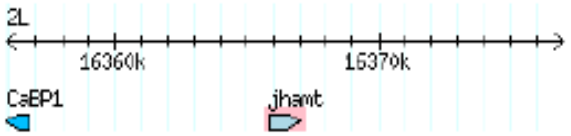
tcgag- 3′

Regions used to create PCR primers for amplification- xxxxx

B. *JHAMT* gene sequence CG 17330 (995 bp)

Sequence location in the genome- 2L:16,365,919..16,366,977 [+]

Genomic map-



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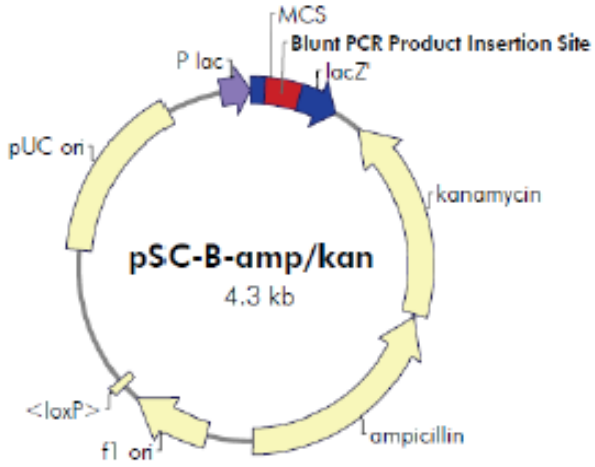
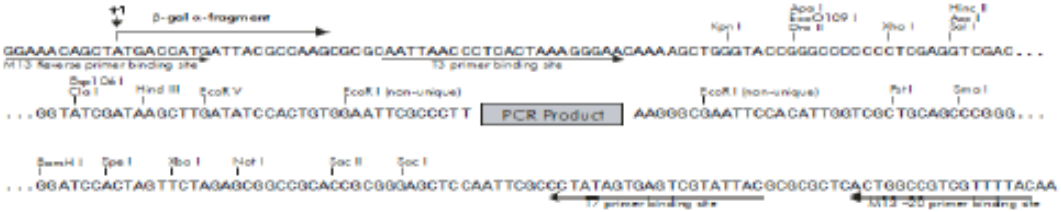
Gene span= xxxx RNA= XXXX CDS=XXXX(FlyBase)

C. pSC-B vector

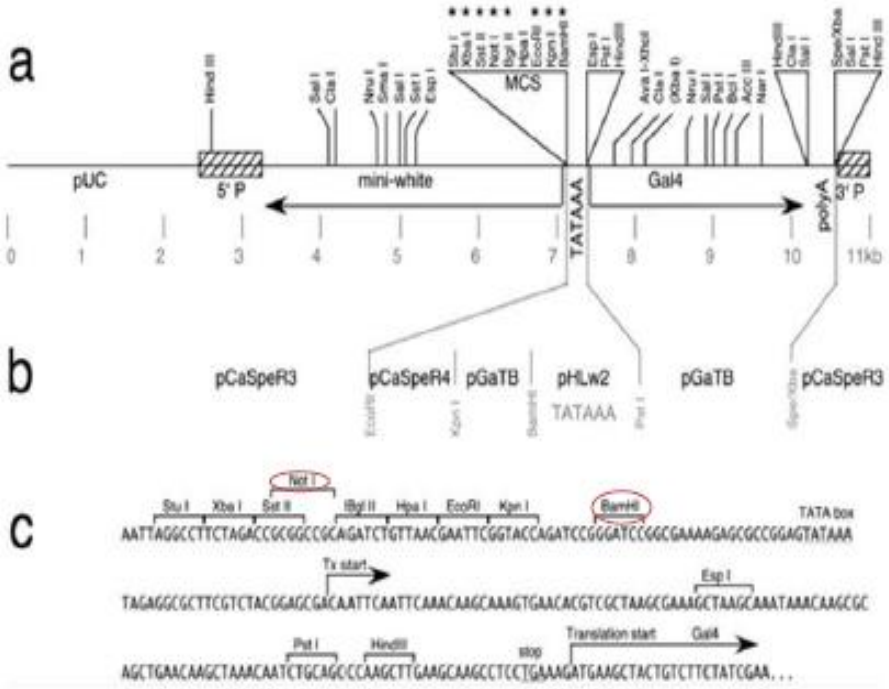


pSC-B-amp/kan Blunt PCR Cloning Vector PCR Product Insertion Site Region
(sequence shown 4263–4272, 1–252)



[StrataClone Blunt PCR Cloning Kit. Instruction Manual]

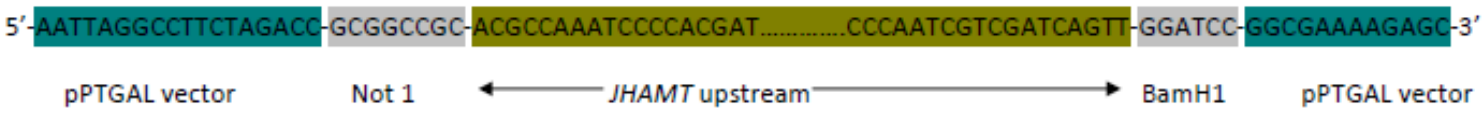
D. pPTGAL vector (*MultiCloning Site of the pPTGAL vector)*



Not1 and BamH1 sites used for insertion of *JHAMT* sequence are indicated.

[Sharma et al., 2002]

E. Alignment of the *JHAMT* upstream promoter region inside the Multicloning site of pPTGAL vector



Appendix 2

A. The *takeout* cDNA sequence used for the construction of the Takeout-GST fusion protein

B. The *takeout* gene sequence

C. pSC-B vector as an intermediate cloning vector to clone the *takeout* cDNA sequence into pAcSecG2T

D. Alignment of the *takeout* sequence inside the pAcSecG2T vector using restriction sites

A. The *takeout* cDNA sequence used for the construction of the Takeout-GST fusion protein

5'-
AAATTCCCCGAAGATCCAAAACCTTGTAAGTATGGTGATGGCGAATGTATCATGAAGCTGTGCAACACCCTGTTCAGCGAAAACTCGGCGGAGGGGGATCC
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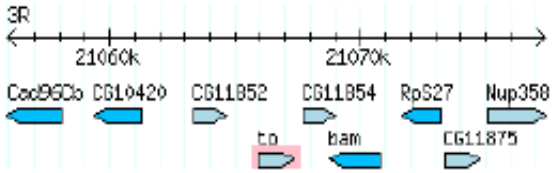
Primer sequences used for amplification- XXXX

B. The *takeout* gene sequence

Sequence location in the genome- 3R:21,065,902..21,067,390 [+]

Genomic map-



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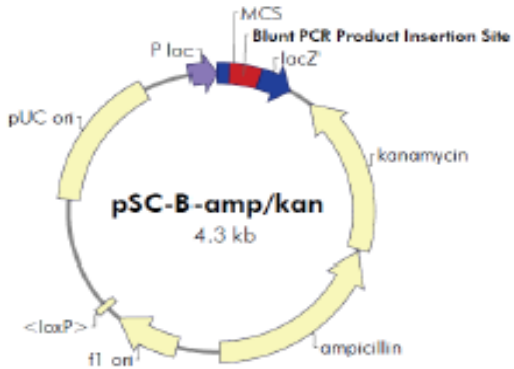
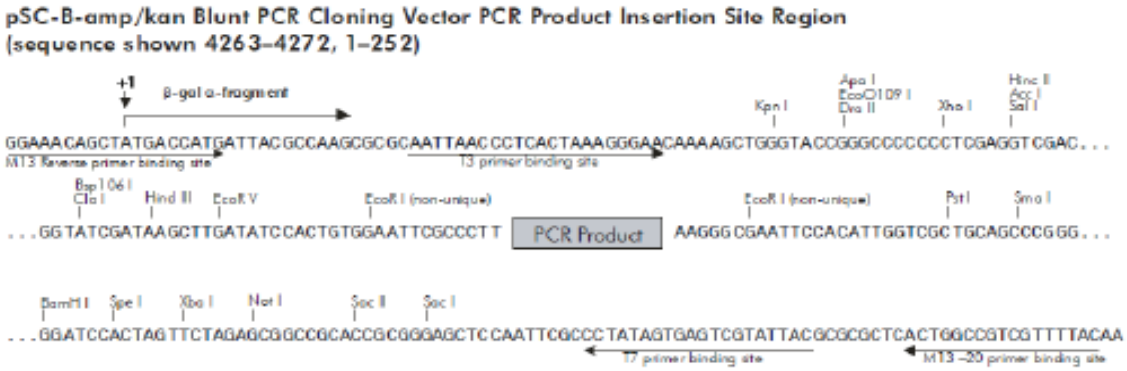
AAAAAGCTTGATAAACAATCTTTGTTGACTTAGCCATTAATTAGACGTTGAAACGGG- 3'

Gene span= xxxx RNA= XXXX CDS=XXXX Signal peptide sequence= XXXX (FlyBase)

C. pSC-B vector as an intermediate cloning vector to clone the *takeout* cDNA sequence into pAcSecG2T

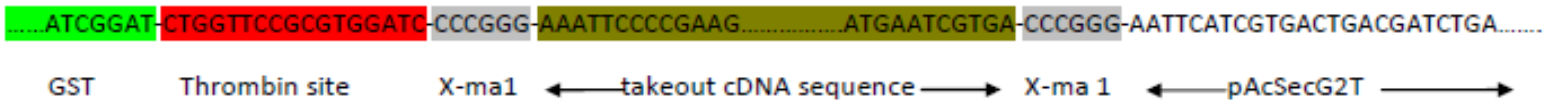
Map for the StrataClone Blunt PCR Cloning Vector pSC-B-amp/kan



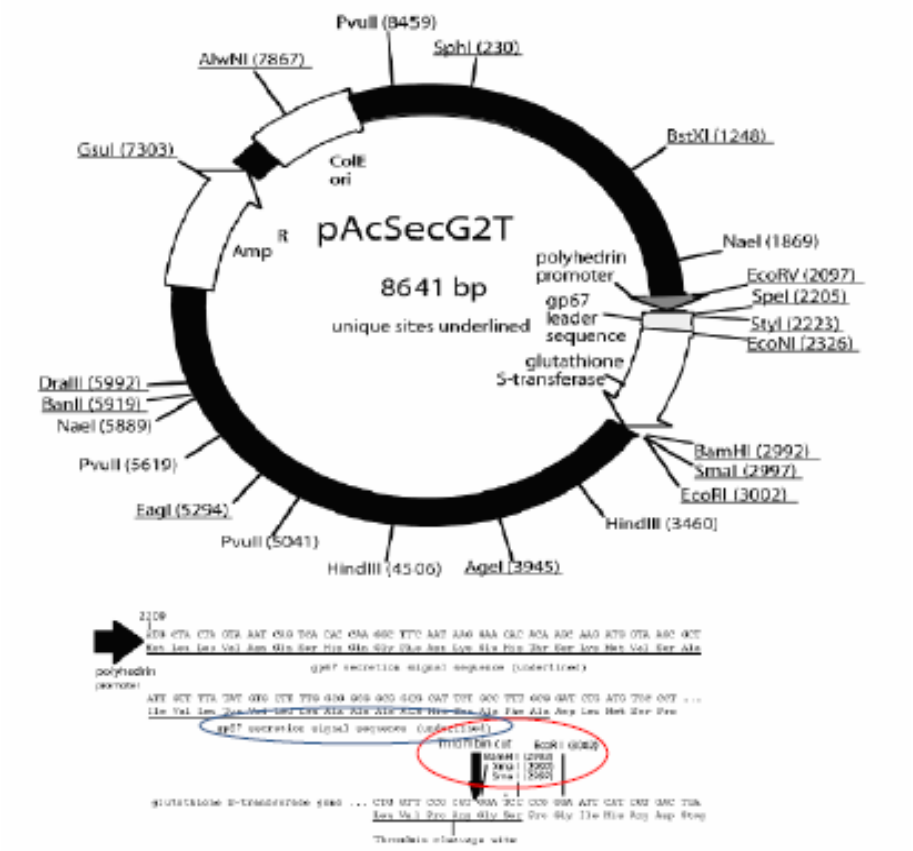


[StrataClone.. Agilent Technologies]

D. **Alignment of the takeout sequence inside the pAcSecG2T vector using restriction sites**



MultiCloning site of the pAcSecG2T vector



gp67 signal sequence-

Multicloning Site of pAcSecG2T-

The pAcSecG2T Baculovirus GST-fusion expression vector. Foreign genes are inserted downstream of the GST coding region into one of the restriction enzyme sites. The *gp67* secretion signal sequence preceeding the GST sequence functions to secrete the recombinant GST fusion protein out of the cell. The *gp67* region is cleaved off from during secretion. GST has a high affinity for reduced glutathione and allows purification by Glutathione Agarose beads. The GST tag is removed from the fusion protein using Thrombin.

Appendix 3

Takeout-GST fusion protein production by Allele Biotechnology Inc.

1. **Takeout protein production in Tni cells.**

Culture 1 L of Tni cells in suspension to 1e6 cell/ml.Plate ~33 plates of Tni cells; 30 ml of cells per 150 X 25 mm Phoenix Biomedical Tissue Culture Dish. Remove and refresh with serum free media containing Takeout BV (MOI= 5) Incubate 27C for 72 hrs. Harvest virus conditioned serum free Tni cell media.

2. **Ammonium sulfate precipitation of Takeout protein**

Centrifuge virus conditioned media for 15' at 5K x g to remove cell debris. *Target Protein in serum free media can't bind to Glutathione affinity beads.* Ammonium sulfate precipitation performed by adding solid to 55-60% (w/v) while stirring on ice (460g per liter of conditioned media).Precipitates harvested by centrifugation at 17,000 x g in a fixed angle rotor for 30 min at 4 C.

3. **GST purification of Takeout protein**

Redissolve crude precipitates in 40 ml of column buffer (20 mM Tris pH 8.0, 250 mM NaCl, 1% TX 100, 1mM DTT) and dialyze overnight against 2L of same buffer. Equivalate Glutathione beads (2 ml bed volume) in column buffer (2 washes; 2 X 25 ml). Combine beads and dissolve crude prep in 50 ml conical tube and place tube on a rocker overnight at 4C.Centrifuge 700g for 4 min to pellet beads.

Save supernatant and label as non bind.

Wash beads with wash buffer 20 mM Tris pH 8.0, 250 mM NaCl, 1% TX 100, 1mM DTT (3 X 50 ml wash; pellet 500g for 3 min). Load/pack beads in last wash into a column; gravity drain. Elute with 3 bed volume of elution buffer (5mM reduced glutathione in wash buffer).

Analyze starting material, non bind, washes, elutions on SDS PAGE

Dialyze the eluted samples in PBS or Tris buffer with 10% glycerol.

REFERENCES

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