UNIVERSITY OF CALIFORNIA
RIVERSIDE

Ecdysis Triggering Hormone Signaling in Adult *Drosophila melanogaster.*

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of the requirements for the degree of

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in

Entomology

by

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DEDICATIONS

I would like to dedicate this dissertation to my dad Mr. Anantprakash Abaji Deshpande, mom Mrs. Jyoti Anantprakash Deshpande, brother Dr. Sarin Anantprakash Deshpande, sister-in-law Dr. Monisha Sharma and my dear friend Mr. Sanjay Pal for their unending support and unconditional love.

"Om Shri Sai Nathay Namah"

"There is no such thing as a problem without a gift for you in its hands.

You seek problems because you need their gifts."

-Richard Bach

ABSTRACT OF THE DISSERTATION

Ecdysis Triggering Hormone Signaling in Adult *Drosophila melanogaster.*

by

Sonali Anantprakash Deshpande

Doctor of Philosophy
Graduate Program in Entomology
University of California, Riverside, March 2012
Dr. Michael E. Adams, Chairperson

Ecdysis triggering hormone (ETH) is a peptide hormone that regulates the behavioral sequence necessary for shedding of exocuticle at the end of each developmental stage, a process called ecdysis. ETH is secreted by Inka cells of the epitracheal glands of insects. *Drosophila* larvae have seven pairs of Inka cells. ETH acts on its receptors (ETHRs), present in the central nervous system (CNS) to regulate the behavior. Ecdysis is a stage specific behavior that does not persist in adults. Interestingly, Inka cells producing ETH are present in the adult stage of *Drosophila*, suggesting a possible role for ETH signaling in adults. Recently, ETHR transcripts were found to be expressed in corpora allata of fourth and fifth instar *Bombyx* larvae. Molting hormone,

20-hydroxy-ecdysone (20HE) and juvenile hormone (JH), through their morphogenetic action are key regulators of insect molting. In adults, they play roles in courtship and reproduction.

In this study, I describe expression of ETH in Inka cells of the adult stage. I further investigated roles of ETHRs in adult *Drosophila melanogaster* behavior by performing RNAi, using the *Aug-21-Gal-4* driver. Although *Aug-21-Gal-4* is reputed to be a corpora allata (CA) specific driver in larval stage it also drives expression in larval salivary glands and gut. In adults, apart from CA, salivary glands and gut, *Aug-21-Gal-4* also drives expression in neurons of the brain and thoracic ganglion. Two pairs of neurons in the brain were identified to be eclosion hormone (EH) neurons and giant fiber neurons. Silencing of ETHRs using *Aug-21-Gal-4* results in elevated male-male courtship, whereas silencing of ETHRs using *EH-Gal-4* or *A307-Gal-4* does not elevate male-male courtship. Elevated male-male courtship was observed after driving ETHR-RNAi using pan-neuronal *elav-Gal-4* and *FruM-Gal-4* drivers. These results indicate that ETHRs in *fru* neurons regulate male courtship behavior. ETHR silencing does not affect female behavior, which can be explained by absence of ETHR transcripts in adult females.

Transcriptome analysis of CA, heads and whole flies was done to check for differentially expressed genes resulting from ETHR-RNAi. Genes involved in male courtship behavior, axon guidance, transcription factors and courtship song are discussed in detail. Chromatin organization genes were downregulated in heads after ETHR-RNAi.

Clustering of differentially expressed genes was observed on chromosomes, indicating a functional role for ETHRs in chromatin organization. Interestingly, male accessory gland specific genes were found to be differentially expressed in male corpora allata tissue. JH expression is known to affect male accessory gland proteins in accessory glands. Presence of male accessory gland specific genes in corpora allata indicates a possible role for JH in regulation of male accessory gland proteins. Male courtship related genes differentially expressed in response to ETHR-RNAi include *doublesex (dsx),* one of the sex determination genes. Comparison of differentially expressed genes from three tissues indicates tissue specific regulation of genes in each tissue. In summary, this is the first study to show a role for ETHRs in adult *Drosophila melanogaster*.

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List of Abbreviations

ETH	Ecdysis Triggering Homrone
ETHR	Ecdysis Triggering Homrone Receptor
CNS	Central Nervous System
CA	Corpora Allata
MAG	Male Accessory Gland
TF	Transcription Factors

CHAPTER I

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

Peptides have long been known to play pivotal roles in behavioral, immune and neuroendocrine systems. Peptide signaling has been evolutionarily conserved over a wide range of organisms. Various organisms utilize peptide signaling to regulate behaviors such as communication and mating. Neuropeptides are utilized in simple organisms like *Hydra* for development, reproduction and communication (Takahashi et al. 2008). Studies in yeast show roles for peptidergic signaling in pheromone communication and mating in single cell organisms (Gooday 1974; Stőtzler et al. 1976). Peptides also are utilized by other higher organisms for many purposes. Invertebrates such as insects utilize peptidergic signaling for communication, defense and behaviors like molting, courtship and mating (Vezenkov et al. 2009). In the last decade, peptides have been studied in various insect model systems as a part of neuroendocrine systems that regulates behaviors (Nässel et al. 2010).

Neuropeptides

Neurons use a variety of chemical messengers for communication. This includes various small molecules, such as glutamate, gamma-aminobutyric acid (GABA), monoamines such as dopamine, octopamine, serotonin, and gases such as nitric oxide, carbon monoxide and various neuropeptides. Neuropeptides are the largest class of signaling molecules. They play major roles in neuronal communication and signaling underlying behaviors. They bind to specific receptors and regulate downstream cascades.

Some neuropeptides regulate specific types of behavior, whereas others are more general neuronal regulators. Some interneurons in the CNS also release neuropeptides and participate in neuronal signaling.

Insect neuropeptides have been a topic of interest for many years. Studying insect neuropeptides provides knowledge that may be useful in developing insect control strategies and treating human diseases. Due to a significant number of genes shared by *Drosophila* and humans, *Drosophila* has long been used as a model organism for studying various human diseases and neuropeptides related to it.

Drosophila **Peptides**

In *Drosophila,* about 2% of all larval CNS neurons are peptidergic. Most of these neurons are present in the brain and ventral nerve cord. Various peptides including adipokinetic hormone (AKH), allatostatins (AST), bursicon, corazonin, crustacean cardioactive peptide (CCAP), diuretic hormone, ecdysis triggering hormone (ETH), eclosion hormone (EH), FMRFamides, kinins, neuropeptide-like precursors, pigment-dispersing factor (PDF), proctolin, and prothoracicotropic hormone have been associated with regulation of various developmental and adult behaviors (Zitnan et al. 2007; Nässel et al. 2010). These neuropeptides are secreted by variable numbers of neurons. For example only two neurons express EH, whereas about 40-50 neurons express CCAP (Kim et al. 2006). In *Drosophila*, about 119 genes encode peptides, of which 43 are annotated. Recently, 76 putative secretory peptide genes were added to the *Drosophila*

melanogaster genome (Liu et al. 2006). This high number of genes coding for peptides in *Drosophila* shows the importance of peptides in insects.

Neuropeptide Receptors

Most neuropeptides interact with G-protein coupled receptors (GPCRs), which regulate cellular responses. GPCRs are seven transmembrane proteins, which after ligand binding, go through a conformational change and activate a G-protein. Conformational changes in GPCRs result in a switch of bound GDP to GTP. As a result, the G-protein dissociates into α and βγ subunits, leading to one of the several downstream cascades. Depending on the G-protein subtype, the result can be elevation of intracellular calcium (Gαq), elevation of cAMP (Gαs) or decreased cAMP (Gαi). Some neuropeptide receptors are guanylate cyclases, which after activation convert GTP to cGMP, while some are receptor tyrosine kinases, which are activated by phosphorylation of receptor tyrosines by ATP (Chang et al. 2009; Lemmon et al. 2010). Detection of second-messenger molecules like cGMP or cAMP by various *in vitro* cell-based assays provides a measure of receptor activation (Kim et al. 2006).

In *Drosophila*, about 2% of the genome encodes GPCRs and about 45 GPCRs have been identified, out of which 15 are orphan receptors. A few exceptions like EH and prothoracicotropic hormone (PTTH) act on a guanylate cyclase and tyrosine kinase, respectively (Brody et al. 2000; Hewes et al. 2001; Chang et al. 2009; Rewitz et al. 2009). *Drosophila* has been used as a model system for studying various types of peptide

regulated behaviors, recently reviewed in detail. (Nässel et al. 2010). Due to presence of 77% of human disease genes in *Drosophila,* this organism has been used for studies of various human diseases like Parkinson's, Alzheimer's etc. and also cellular processes such as learning and memory (Reiter et al. 2001). Neuropeptides in *Drosophila* regulate molting, feeding, locomotion, olfaction, learning and social behaviors like courtship and aggression (Nässel et al. 2010).

Ecdysis

Ecdysis, or shedding of exocuticle, is one of the most important behaviors that insects perform during their development. At the end of each molt, insects shed their cuticle by performing a particular sequence of body movements, which includes pre-ecdysis, ecdysis and post-ecdysis behaviors. It is an innate behavior, which is regulated by a wide range of hormones including various peptides like EH, ETH, crustacean cardio-active peptide (CCAP), FMRFamide, kinins, myoinhibitory peptide (MIP) and bursicon. This cascade of peptides regulates the ecdysis behavioral and physiological sequence, allowing the insect to shed its exocuticle (Zitnan et al. 2007). The sequence of events and roles of these peptides have been well studied in insects, including *Drosophila*.

The ecdysis behavioral sequence is activated by the peptide hormone, ecdysis triggering hormone (ETH), which is produced by Inka cells of the epitracheal gland. ETH acts on its receptors (ETHRs) located in the CNS to regulate the behavior. Most ETHR

neurons are peptidergic and release peptides as a result of activation by ETH. The process of ecdysis is critical for insect development; any variation in scheduling of the behavior can be fatal.

Ecdysis Triggering Hormone

Growth and development of insects depends on periodic shedding of exocuticle through ecdysis. ETH acts as a command chemical to orchestrate the ecdysis behavioral sequence in insects (Kim et al. 2006). It is secreted by Inka cells present in epitracheal glands (Zitnan et al. 1996; Park et al. 1999; Zitnan et al. 2002; Zitnan et al. 2003). In *Drosophila melanogaster,* the ETH gene encodes two subtypes of ETH called ETH-1 and ETH-2. The ETH precursor is a short peptide with 203 amino acids. This peptide hormone is conserved in insects and has been studied in *Anopheles, Bombyx, Culex, Drosophila, Manduca, Tribolium* etc. (Roller et al. 2010). Surprisingly, in *Drosophila* Inka cells persist in the adult stage, where ecdysis behavior is absent (Fig. 1-1) (Park et al. 2002). Recent RNA-seq results also show the presence of *ETH* transcripts in adult stages (Fig. 1-2) (Graveley et al. 2011). This raises the question about the possible functional roles of ETH signaling in the adult stage of *Drosophila.*

ETH release from Inka cells is regulated by two peptide hormones, EH and corazonin and by levels of a steroid hormone, 20-hydroxy-ecdysone (20-HE) (Ewer 1997; Zitnan et al. 1999; Kim 2004). Presence of an ecdysone receptor regulatory element (EcRE) upstream of the ETH gene indicates the likelihood that 20-HE regulates

ETH expression directly (Zitnan et al. 1999). Rising ecdysteroid levels leads to an increase in ETH levels and size of Inka cells. Release of ETH is enabled by decreasing ecdysteroid levels (Kingan et al. 1997). In *Manduca*, exposure of epitracheal glands to EH results in release of ETH this action can be also be mimicked by increasing cGMP levels in the Inka cells (Kingan et al. 2001). Corazonin (CRZ) is another ETH releaser, which acts on corazonin receptors, present in Inka cells. Exposure to low concentrations of CRZ results in the release of ETH from Inka cells (Kim et al. 2004).

Ecdysis Triggering Hormone Receptors

ETH acts on ecdysis triggering hormone receptor (ETHRs) neurons to regulate ecdysis behaviors (Park et al. 2003). ETHR is a 550 amino acid G-protein coupled receptor, which activates the Gαq pathway. ETHR activation can be measured by monitoring intracellular calcium mobilization in target neurons (Kim et al. 2006). In *Drosophila,* two subtypes of ETHRs (ETHR-A and ETHR-B) are encoded by the gene CG5911 through alternative splicing (Park et al. 2003). These splice variants are expressed in mutually exclusive sets of neurons in the CNS (Kim et al. 2006). Separate populations of ETHR-A and ETHR-B neurons suggest they play different roles. Most ETHR-A neurons are peptidergic, while ETHR-B neurons remain largely uncharacterized. ETHR-A is expressed in neurons producing CCAP, kinin, EH, bursicon, FMRFamide and MIP. Each neuronal ensemble was identified by immunostaining and monitoring Ca^{2+} dynamics of neurons after ETH application (Kim et al. 2006).

Targeted ablation of these neurons causes severe changes in pupal ecdysis behavioral scheduling. Cell killing of certain ETHR-A ensembles such as CCAP neurons is lethal, whereas killing other ensembles like EH neurons does not affect ecdysis scheduling (McNabb et al. 1997; Park et al. 2003).

Recent Advances

Recently, ETHR transcripts were detected in corpora allata (CA) of 4th and 5th instar larvae of the silkmoth, *Bombyx mori* (Yamanaka et al. 2008). This gland is responsible for release of juvenile hormone (JH), which promotes juvenile phenotypes during immature stages of development. In adult *Drosophila*, JH controls reproductive processes in adult females. Recent RNAseq data shows the presence of transcripts in 1, 5 and 30 day old adult males, whereas the transcript levels in females are only observed on day 1. Interestingly, ETH transcripts persist in adult females but ETHR transcripts are lacking. Since ETHRs persist in adult stages where ecdysis is no longer observed, it is interesting to ascertain fucntional roles of ETHRs in adult stages.

Adult Behaviors

Adult behaviors such as feeding, locomotion, learning and social behaviors, courtship and aggression have been studied in adult *Drosophila*. Behaviors are complex processes in organisms which are a result of sensory, environmental, mechanical inputs and are regulated by various genes (Siegel et al. 1979; Levine et al. 2002; Carney 2007; Ellis et al. 2009; Ellis et al. 2011)

Courtship Behavior

Courtship behavior, like all behaviors, is influenced by various sensory inputs and regulated by various genes, including sex determination genes, *fru and dsx* (Villella et al. 1996; Demir et al. 2005). In *Drosophila*, males display a stereotypic behavioral sequence, which includes orientation, tapping, singing, licking, attempt and copulation (Greenspan et al. 2000). Male behaviors are influenced by female pheromones and inhibited by the presence of male pheromones (Whalley 2007). Females respond to male singing or wing extension, also known as "courtship song", which is an acoustic signal produced by wing vibration (Greenspan et al. 2000). A receptive female walks away from the male and is followed by the male. The male mounts her and attempts to copulate. Non-receptivity of a female is displayed by protrusion of the vaginal plates. During mating, males transfer the male specific pheromone, cis-vaccenyl acetate (cVA) to the female, making her non-attractive for other males (Ejima et al. 2007).

Recently, genetic approaches have been used to investigate cellular bases for behavior. Behavior is known to alter gene expression and vice-versa (Carney 2007; Ellis et al. 2009; Ellis et al. 2011). Courtship behavior in *Drosophila* is a result of genetic and social interactions (Greenspan et al. 2000; Billeter et al. 2002). Mutations of genes, including sex-determination genes *fru* and *dsx,* courtship song genes, *cac,* and sensory genes (Smith et al. 1998), *Or67d, Or65a, Gr33a, Gr32a, Gr68a* are known to affect male courtship behavior (Bray et al. 2003; Kurtovic et al. 2007; Miyamoto et al. 2008; Moon et al. 2009). Single gene mutation studies have been done to check the effect of a gene on

various behaviors. Unfortunately, little is known about changes in gene expression as a result of these mutations, which leads to behavior.

The primary focus of this dissertation is to explore possible roles for ETHRs in adult behaviors. In Chapter 2, receptors were silenced using the RNAi technique. The Gal-4/UAS system was used and adult behaviors were analyzed. Adults were tested for fecundity and behaviors including courtship, mating, and aggression. Genes are known to be regulated differently in various tissues. In order to compare altered patterns of gene expression, three samples were used for transcriptome analysis. In Chapter 3, transcriptome analysis of corpora allata, heads and whole flies was done using RNAseq analysis on Illumina platform. To determine differentially expressed genes, expression levels of genes from ETHR-RNAi and control tissues were compared. Differentially expressed genes were subject to further analysis and were discussed in details. Outcomes of these investigations led to further hypothesis-testing and a model to explain mechanisms underlying ETHR-regulated behaviors in adult *Drosophila*.

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Figure 1-1. Location of Inka cells shown by immunostaining and EGFP expression in the 2eth3egfp transgenic fly line. Adapted from (Park et al. 2002). **A)** Larval and **B)** Adult tracheal system and positions of Inka cells marked in red.

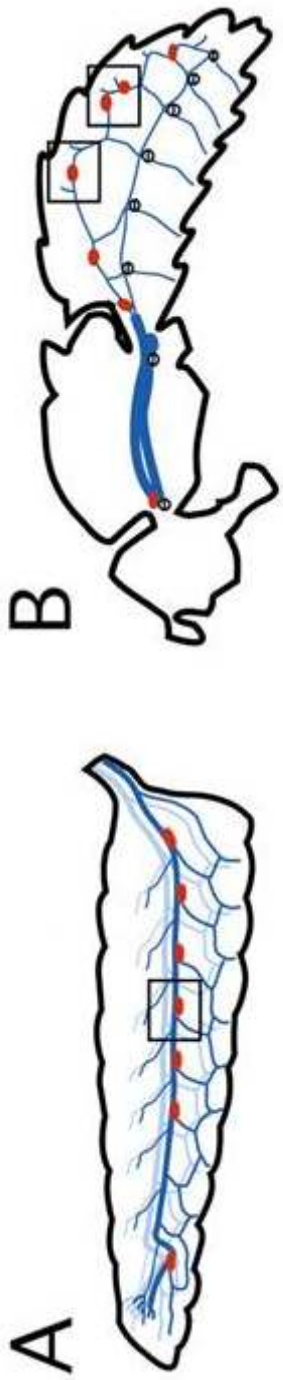
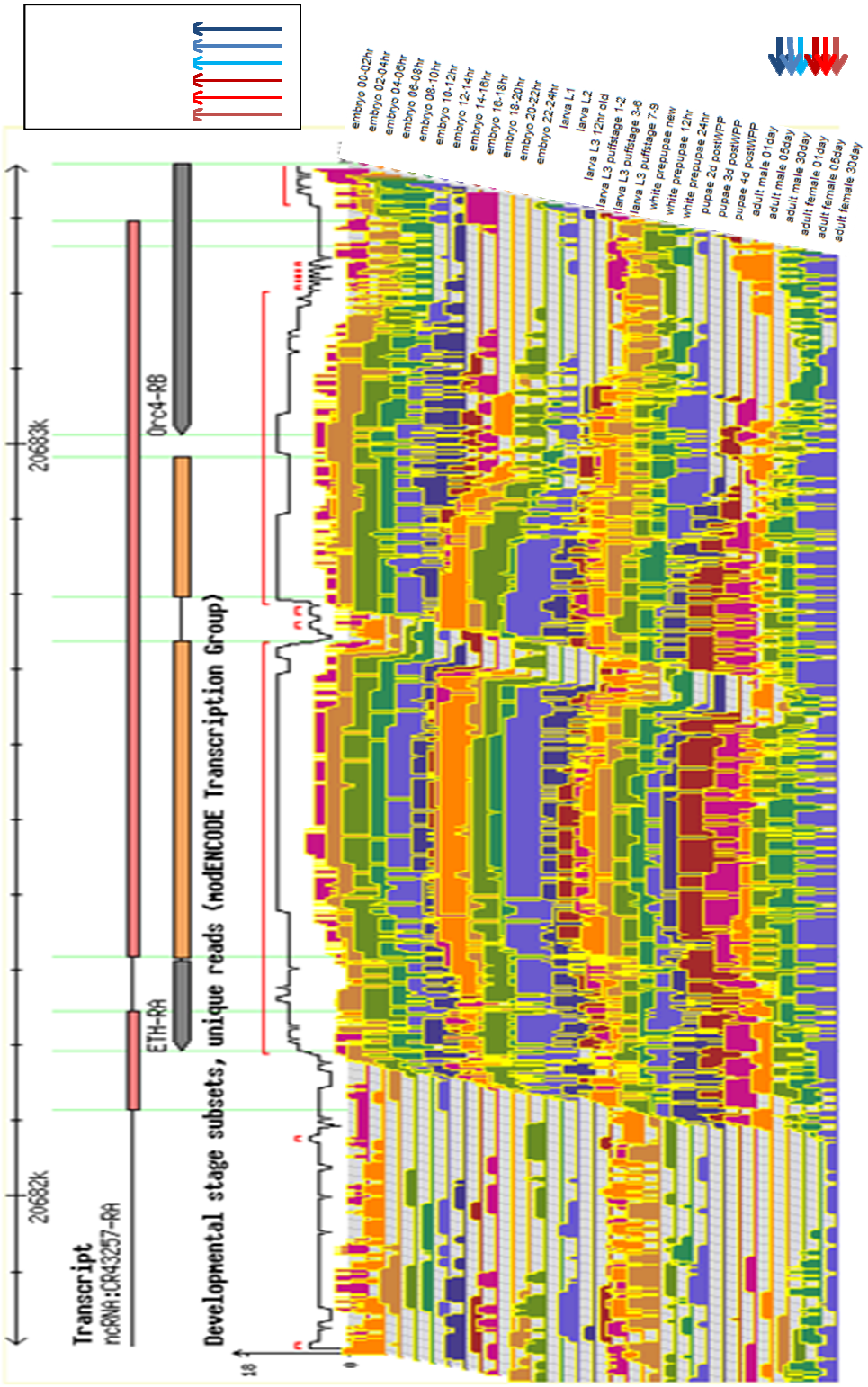


Figure 1-1.

Figure 1-2. Reads from 1, 5 and 30 day old male and female adults mapping ETH gene. Blue arrows indicate adult male adults and red arrows indicate adult females. Figure modified from www.flybase.org ModECODE RNAseq data.


Transcript
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20682k
20683k
ETH-RA
Orc4-RB
Developmental stage subsets, unique reads (modENCODE Transcription Group)
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embryo 02-04hr
embryo 04-06hr
embryo 06-08hr
embryo 08-10hr
embryo 10-12hr
embryo 12-14hr
embryo 14-16hr
embryo 16-18hr
embryo 18-20hr
embryo 20-22hr
embryo 22-24hr
larva L1
larva L2
larva L3 12hr old
larva L3 puffstage 1-2
larva L3 puffstage 3-6
larva L3 puffstage 7-9
white prepupae new
white prepupae 12hr
white prepupae 24hr
pupae 2d postWPP
pupae 3d postWPP
pupae +d postWPP
adult male 01day
adult male 05day
adult male 30day
adult female 01day
adult female 05day
adult female 30day

Figure 1-2.

Figure 1-3. Stage specific ETHR reads show absence of ETHRs in adult females.

Blue arrows indicate adult male adults and red arrows indicate adult females.

Figure modified from www.flybase.org ModECODE RNAseq data.

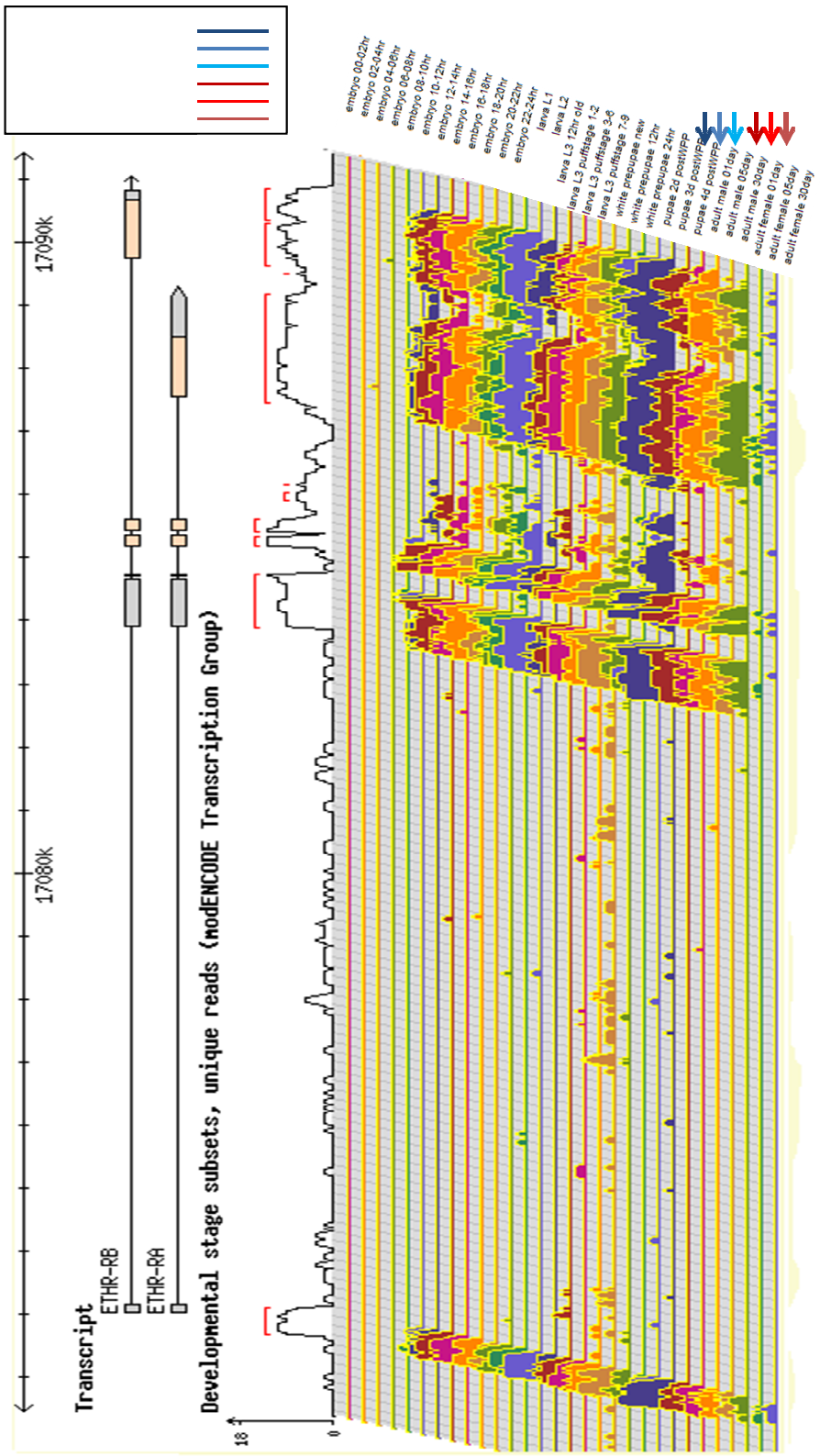
17090k
17080k
Transcript
ETHR-RB
ETHR-RA
Developmental stage subsets, unique reads (modENCODE Transcription Group)
18
0
embryo 00-02hr
embryo 02-04hr
embryo 04-06hr
embryo 06-08hr
embryo 08-10hr
embryo 10-12hr
embryo 12-14hr
embryo 14-16hr
embryo 16-18hr
embryo 18-20hr
embryo 20-22hr
embryo 22-24hr
larva L1
larva L2
larva L3 12hr old
larva L3 puffstage 1-2
larva L3 puffstage 3-6
larva L3 puffstage 7-9
white prepupae new
white prepupae 12hr
white prepupae 24hr
pupae 2d postWPP
pupae 3d postWPP
pupae 4d postWPP
adult male 01day
adult male 05day
adult male 30day
adult female 01day
adult female 05day
adult female 30day

Figure 1-3.

CHAPTER II

Ecdysis Triggering Hormone Receptors in

Drosophila melanogaster Adult Courtship Behavior.

ABSTRACT

Ecdysis triggering hormone (ETH) is a neuropeptide released by Inka cells. This peptide hormone acts on G-protein coupled receptors (ETHRs), leading to calcium release from intracellular stores via the Gαq pathway. ETHRs are present in the central nervous system (CNS), and after activation regulate ecdysis behavior and shedding of exocuticle in insects. Inka cells persist in adults, raising the question of the functional role of ETHRs in this stage. Recent findings in the silkworm *Bombyx mori* show presence ETHRs in the corpora allata (CA), the sole source of juvenile hormone (JH). JH is known to be involved in development, reproduction and diapause in insects. In *Drosophila*, exposure of isolated adult CA to ETH results into calcium mobilization, suggesting that ETHRs occur in adult CA. Here, I show that ETHRs in adult *Drosophila* are involved in regulation of courtship behavior in adult males, because silencing ETHRs increases male-male courtship. Silencing ETHRs in females does not affect behavior or fecundity. This is the first ever function shown for ETHRs in adult flies.

INTRODUCTION

Ecdysis triggering hormone (ETH) is a peptide hormone, which acts as a command chemical to trigger a behavior sequence critical for shedding of exocuticle in insects. The ETH gene is conserved in various insect species including, *Drosophila melanogaster* (Park et al. 1999), *Aedes aegyptii* (Dai et al. 2009), *Tribolium casteneum* (Amare et al. 2007), *Apis mellifera* (Hummon et al. 2006), *Bombyx mori* (Zitnan et al.

2002), *Manduca sexta* (Zitnan et al. 1996), *Locusta migratoria* (Clynen et al. 2006) etc.(Zitnan et al. 2003). Recently, ETH and ETHRs were found in the crustacean, *Daphnia pulex* (Christie et al. 2011). This neuropeptide is released from the Inka cells present on the epitracheal glands and plays an important role in regulating ecydsis behavior (Roller et al. 2010). Ecdysis is an innate behavior, whereby the insect performs specific body movements in order to loosen its cuticle and eventually shed it. Deletion of the ETH gene (*ETH*) in *Drosophila* results in ecdysis related deformities and lethal phenotype (Park et al. 2002). Surprisingly, Inka cells persist in the adult stage of *Drosophila melanogaster*, a stage where no further ecdysis occurs.

ETH acts on its receptors (ETHRs) in the central nervous system (CNS) to regulate a peptide signaling cascade that schedules ecdysis related behaviors (Kim et al. 2006). ETHRs are G-protein coupled receptors (GPCRs), which activate the Gαq pathway to release calcium downstream from intracellular stores. In *Drosophila,* two alternative splice variants of the gene ETHR gene (*ETHR*) encode ETHR-A and ETHR-B. These two receptor subtypes are expressed in mutually exclusive populations of neurons. Most ETHR-A neurons are peptidergic. These neurons release various peptides downstream of ETH signaling forming a cascade, which leads to ecdysis behavior. (Kim et al. 2006). These receptors are also conserved in insects including *Drosophila melanogaster, Aedes aegyptii, Tribolium casteneum, Apis mellifera, Bombyx mori, Manduca sexta, Locusta migratoria* and a crustacean *Daphnia pulex* (Roller et al. 2010). In *Drosophila,* ETHR expression persists in adult males but not in females. Presence of

ligand and no receptor in adult females could indicate presence of unidentified receptors for ETH.

Recently, ETHR transcripts were detected in corpora allata (CA) of fourth and the fifth instar larvae of silkworm, *Bombyx mori* (Yamanaka et al. 2008). The CA are responsible for release of juvenile hormone (JH). JH is a sesquiterpenoid which regulates of development, reproduction and diapause in insects. JH promotes juvenile phenotypes in immature stages of development (Truman et al. 2007). In adult *Drosophila,* JH regulates oogenesis (Soller et al. 1999), neuroendocrine stress reactions (Rauschenbach et al. 1995) and male courtship behavior (Liu et al. 2008). Presence of ETHRs in CA indicates their possible involvement in JH release and JH regulated behavior. In *Drosophila* adults, exposure of isolated CA to ETH results in calcium elevation, indicating the presence of ETHRs in *Drosophila* adult CA.

In this study, I investigated possible functional roles for ETHRs in adult *Drosophila*. If ETHRs are involved in JH release in adults, silencing ETHRs could affect JH related adult behaviors. In order to silence ETHRs in *Drosophila* CA, ETHR RNAi was done using a known CA specific Gal-4 line and adult behaviors were compared to control flies. Fecundity and innate behavior including courtship and mating were measured. In *Drosophila*, males perform a stereotypic behavior as a courtship ritual. This innate behavior includes following the female, orientation, wing extension (also known as courtship song), licking the female abdomen, and attempted copulation (bending of abdomen) (Spieth 1974; Hall 1982; Hall 1994; Greenspan et al. 2000). Courtship

behavior is known to be under the control of a wide variety of hormones, including juvenile hormone and ecdysone (Liu et al. 2008; Ishimoto et al. 2009).

MATERIALS AND METHODS

Insect Rearing. *Drosophila melanogaster* were reared on regular cornmeal medium at 25ºC under 12:12 light: dark cycle. *ETH-Gal-4* and *symUAS-ETHR-RNAi* flies were obtained from our lab stock, wCS (cantonized *W1118*) flies were gifted by Dr. Dahanukar, *Aug21-Gal-4/cyo* flies were gifted by Dr. Korge, *fru-Gal-4 and dsx-Gal-4*$^{(1)}$ lines were gifted by Dr. Baker and all other flies were obtained from Bloomington stock center. Canton-S (CS) flies were used as wild type for cantonized flies and *W1118* flies were used as controls for non-cantonized flies. Flies with *W1118* background were labeled as (w) and cantonized flies were labeled as (cs). *Aug21-Gal-4/+, symUAS-ETHR-RNAi/+* flies were obtained by crossing *Aug21-Gal-4/cyo* and *symUAS-ETHR-RNAi* respectively with wild type flies. *Aug21-Gal-4/+*(Gal-4 only)*, symUAS-ETHR-RNAi/+*(UAS only)*, Aug21-Gal-4/symUAS-ETHR-RNAi* (RNAi) or wild type flies were used as test flies and wild type flies were used as subject. Flies were collected within 12 hrs after eclosion under CO_2 anesthesia. Males were individually aged after collection in 12 X 75mm pyrex glass culture tubes (Corning, NY, United States) with about 1.5cm food at the bottom whereas, virgin females were aged in groups of 5-7 per vial.

***ETH* expression.** *ETH* expression in male and female adult *Drosophila* was determined using *ETH-Gal-4/UAS-CD4-tdTomato* flies at day 1, 5 and 15 adult stages. Flies were

immobilized on ice and internal tissues were dissected to expose the tracheal system. Slides were prepared after fixing the tissue with 4% paraformaldehyde.

Immuno-staining. Expression pattern of *Aug-Gal-4* fly line was done by crossing it with *UAS-EGFP* flies and dissecting CNS along with CA and gut and staining the tissue against anti-GFP. All the tissue samples were dissected in phosphate saline buffer and were stained using standard staining protocols. Samples were immediately fixed by transferring in to 4% paraformaldehyde and stored at 4ºC overnight. After 3, 5min washes with PBST (0.2% PBST: 50ml PBS + 100µl Triton X 100), 5% normal goat serum in PBST was used for blocking at 4ºC overnight. Tissue samples were washed 3 times with PBST and were incubated with primary antibody, Rabbit anti-GFP in the ratio of 1:2000 and stored at 4ºC overnight. After 3 washes with PBST, tissue samples were incubated with the secondary antibody, Alexa 488 Goat anti-Rabbit at 4ºC overnight. Further, 3 washes with PBST were followed by 1 wash with PBS and samples were exposed through a series of gradually increasing concentrations of glycerol solutions. Tissue samples were mounted on a glass slide using 100% glycerol. Confocal images were taken at the Institute for Integrative Genome Biology, University of California, Riverside, on the Leica SP2 confocal microscope.

Video Recording. Videos were recorded using Sony HDR-XR150 for 10 mins and behavior analysis was done manually. Video analysis was done on Toshiba DVD recorder (model RD-XS35) by slowing down the video by 16x. All the data analysis was

done blind. All the data was recorded and analyzed completely randomly across the genotype.

Courtship Assays. Courtship assays were performed using 48-well polystyrene plate with chamber dimensions of 15 mm height and 10 mm diameter. One test and one subject fly was carefully aspirated into the arena and a maximum of six pairs were recorded at a time. Courtship behavior included following, orientation, tapping, singing and attempt (bending of abdomen). Courtship index (CI) for male-male courtship was calculated as percentage of time spent courting other male in 10 mins. Whereas, in case of male-female courtship, courtship index was calculated as percentage of time spent by a male courting a female either until copulation or 10 mins if there was no copulation. Wing-extension index (WEI) was calculated as the percentage of time a male spreads its wing perpendicular to its body during 10 mins of interaction time. A total of 10-20 pairs were tested for each genotype and an average CI and WEI was calculated and standard error mean (SEM) was determined for each genotype. All the courtship assays were performed between Zeitgeber time 7 to 9 at 25ºC (Goldman et al. 2007).

Aggression Assays. Aggression assays were performed in individual chambers of 12-well polystyrene plates with chamber dimension was 21 mm diameter 18 mm depth, containing a food cup made of the cap of a 1.5 ml Eppendorf tube. Flies were aspirated in pairs as one CS and one test genotype. Experiments were started at Zeitgeber time 1 at 25ºC. (Fernández et al. 2011) A total of 10 pairs of flies were tested for aggression behavior and an average number of lunges were calculated for each genotype.

Courtship Latency. Courtship latency was determined by carefully aspirating one virgin test female or test male and one CS male into the behavior chamber. Courtship latency was calculated as time taken by a male to initiate courtship after pairing. (Bray and Amrein 2003) In case of male- male interaction, courtship latency was calculated for test male against CS male and in case of male-female interaction, courtship latency was calculated for CS male against test female. 10 cantonized males were tested individually against CS females and 10 CS males were tested against virgin test females.

Mating Latency. Mating latency was determined by carefully aspirating single male and a female into the behavior chamber. Mating latency was calculated as time from the start of the courtship to mating. 10 cantonized males were tested individually against CS females. Males were carefully aspirated individually with virgin CS female into the behavior chamber and pairs were recorded for maximum of 10 mins. Pairs that did not mate before the end of 10 mins were considered non-mating pairs. Fly pairs that did not mate were taken out from the analysis and final mating latency was represented as an average of all the mated pairs. (Bray et al. 2003)

Receptivity. Female receptivity was calculated by aspirating single male and a virgin female into the behavior chamber. The fly pairs were allowed to interact and their behavior was recorded for 10 mins. Females were considered receptive if they mated within 10mins. Percentage of females mated was calculated out of the 10 pairs tested.

Fecundity. For fecundity, 5 virgin females and 5 wild-type (Canton-S) males were paired in a 35X10mm petri dish (BD Falcon, Fisher Scientific) with regular food at the bottom. Flies were transferred to fresh food dish after every 24hrs by temporarily cooling flies on ice and numbers of eggs were averaged for 5 females. Experiment for each genotype was done in triplicates.

Statistics. Statistical analyses were performed on all data sets using (http://faculty.vassar.edu/lowry/VassarStats.html). The Kruskal-Wallis test with Mann-Whitney *post hoc* test was performed on non-parametric data sets for statistical comparison of behaviors exhibited by ETHR silenced flies and control flies. WEI of *fru-Gal-4/ETHR-RNAi* and *dsx-Gal-4/ETHR-RNAi* were statistically compared to *W1118* WEI using the Student's t-test.

RESULTS

Thoracic ETH Expression Persists in Adults. *ETH-Gal-4/UAS-CD4-tdTomato* flies, both males and females, were dissected at pharate adult, 1 day, 5 day and 15 day old adult stages. Detection of the tdTomato serial indicated that *ETH* expression is present in the thorax of both male and female adults from all stages (Fig.2-1). Five animals in each stage, from both sexes were dissected carefully to expose tracheal tubes and slides were prepared after fixing the tissue. Amongst all the animals checked, tdTomato expression was only observed in two pairs of thoracic Inka cells none of the animals exhibited *ETH* expression in abdominal Inka cells.

Immuno-staining of *Aug-21-Gal-4* Shows Widespread Expression in the CNS. Expression patterns in CNS, including CC and CA of *Aug-21-Gal-4* lines was observed after crossing flies with *UAS-EGFP* flies and staining with rabbit anti-GFP and goat anti-rabbit antibodies. Confocal images were taken and expression patterns were used to choose Gal-4 lines for driving ETHR-RNAi. The expression pattern of *Aug-21-Gal-4/UAS-EGFP* indicated staining in a subset of central neurons, including giant fiber and eclosion hormone neurons (Fig. 2-2a). *Aug-21-Gal-4* is reputed to be a CA specific driver in larval stages (Siegmund et al. 2001; Colombani et al. 2005). However it also drives expression in larval salivary glands and gut. Based on their positions, two pairs of neurons were identified to be eclosion hormone and giant fiber neurons (Fig. 2-2b-d). Gal-4 drivers for EH and giant fiber neurons were used for further analysis. Aug21-Gal-4 was suspected to drive expression in allatostatin-C (Ast-C) neurons. Double staining using Ast-C antibody in *Aug-21-Gal-4/UAS-CD4-GFP* flies shows no overlap in expression of Ast-C and *Aug-21-Gal-4* (Fig. 2-2e).

***Aug-21-Gal-4* Driven ETHR-RNAi Elevates Male-Male Courtship.** Male-male courtship was increased after silencing ETHRs. ETHR silenced (RNAi) males with the genotype *Aug21-Gal4/symUAS-ETHR-RNAi*, show significantly ($p < 0.001$) higher courtship index (CI) with an average ± SEM of 35.75±5.43 for cantonized males towards CS males and 43.38±6.35 for *W1118* background males towards *W1118* males. Both control genotype *Aug21-Gal4/+* (Gal-4 only) males and *symUAS-ETHR-RNAi/+* (UAS only) males show an average CI ± SEM of 10.12±1.18 and 5.50±0.98 respectively, for

cantonized lines towards *Canton-S* (CS) males and 4.5±0.67 and 3.78±0.80 respectively, for *W1118* background males towards *W1118* males. This CI was not significantly different from CI of their respective wild-type CS males (8.32±1.09) towards CS males and *W1118* males towards *W1118* (10.39±2.30) (Fig. 2-3 a-b).

RNAi males also were tested against different genetic background wild-type control males and themselves (RNAi males). *Aug21-Gal4/symUAS-ETHR-RNAi(CS)* males show significantly higher CI, with an average ± SEM of 22.5±2.32 towards *W1118* males and 24.40±4.63 towards other RNAi(cs) males. Similarly, *Aug21-Gal4/symUAS-ETHR-RNAi(w)* males show an elevated CI with an average ± SEM of 19.33±2.87 towards CS males and 20.11±2.16 towards other RNAi(w) males.

<u>*elav-Gal-4* and *fru-Gal-4* Driven ETHR-RNAi Also Elevates Male-Male Courtship.</u>

Based on *Aug21-Gal-4/UAS-EGFP* immunostaining in *Drosophila* adults, it is clear that the *Aug-21-Gal-4* line is not specific for CA in the adult stage. *Aug-21-Gal-4* drives expression in many neurons in the CNS, including paired giant fiber descending neurons and EH neurons. Hence, behavioral changes associated with ETHR-RNAi using *Aug-21-Gal-4* flies cannot be attributed to CA only. In order to specify the tissue location, several other Gal-4 lines were used to drive ETHR-RNAi. These include *elav-Gal-4; tub-gal-80^{ts}*, to drive pan neuronal, temperature controlled expression, in order to avoid any ecdysis defects. *elav-Gal-4/ETHR-RNAi; tub-gal-80^{ts}* males show significantly higher CI, with an average ± SEM of 17.67±3.65 and WEI of 1.46±0.80 towards *W1118*

males. The *A307-Gal-4* (Storkebaum et al. 2009) line was used to drive ETHR-RNAi in the giant neuron system. Courtship behavior of *A307-Gal-4/sym-UAS-ETHR-RNAi* males was tested against *W1118* males. *A307-Gal-4/sym-UAS-ETHR-RNAi* males show no significant difference with *W1118* controls at $p > 0.05$, with an average CI ± SEM of 4.98±1.23 and WEI±SEM of 0.22±0.13. Since ETHR silencing using *Aug-21-Gal-4* causes increased male-male courtship, it is suspected that ETHRs are expressed in *fruitless* or *doublesex* neurons and silencing in those neurons would affect male-male courtship. Hence, *fru-Gal-4*, specific for *fruitless* neurons and *dsx-Gal-4*, specific for *doublesex* neurons (Robinett et al. 2010) were used to drive ETHR-RNAi. The courtship index of *fru-Gal-4/sym-UAS-ETHR-RNAi* males towards *W1118* males was significantly (p-value < 0.05) higher than controls with an average ± SEM of 23.75±7.64 and WEI of 5.73±2.37. *dsx-Gal-4*/sym-*UAS-ETHR-RNAi* males did not show significantly higher CI with an average ± SEM of 4.44±1.36 and WEI of 0.22±0.19 towards *W1118* males (Fig. 2-4).

Aug-21-Gal-4 **Driven ETHR-RNAi Elevates Male-Male Wing Extension Index.**

Courtship behavior can also be described in terms of amount of time a test male extends its wing during courtship behavior, also referred to as wing-extension index (WEI). Similar to CI, WEI of RNAi(cs) males is significantly higher ($p < 0.001$) with an average ± SEM of 7.9±1.72 towards *CS.* Similarly, RNAi(w) flies show significantly higher ($p < 0.001$) WEI with an average ± SEM of 11.34±2.37 towards *W1118* flies . WEI for both Gal-4 only males and UAS only males show an average WEI±SEM of 0.53±0.19 and

0.11±0.05, respectively for cantonized and 0.16±0.03 and 0.07±0.03 respectively, for *W1118* background flies. This is not significantly different than their respective wild types (CS with 0.16±0.08 and *W1118* with 1.19±0.33).

RNAi flies also were tested against wild-type flies of opposite background. RNAi(w) flies courted *W1118* flies at significantly higher rates ($p < 0.001$), with WEI (average ± SEM) of 2.96±1.69 towards CS and 2.93±0.63 towards other RNAi(w) males. RNAi(cs) flies show significantly higher courtship rates ($p < 0.001$), with WEI of 4.31±0.94 towards *W1118* and 4.38±0.90 towards other RNAi(cs) males (Fig. 2-5 a-b).

Wing extension index of *fru-Gal-4/sym-UAS-ETHR-RNAi* males towards *W1118* males was significantly (p-value < 0.05) higher than controls with an average ± SEM of 3.65±1.16. *dsx-Gal-4*/sym-*UAS-ETHR-RNAi* males did not show significantly higher WEI, with an average ± SEM of 0.22±0.19 towards *W1118* males (Fig. 2-5 c).

Aug-21-Gal-4 **Driven ETHR-RNAi Does Not Affect Male Courtship Latency.** ETHR silencing did not affect courtship latency, defined as the amount of time taken by the test male to initiate courtship with the subject male. The average amount of time taken by a test male to initiate courtship with a subject male was not significantly different at $p > 0.1$. RNAi(cs) males show courtship latencies averaging ± SEM of 29.26±4.18, which is not significantly different than Gal-4 only (cs), UAS only (cs) or CS flies; 23.23±5.23, 100.05±21.30 and 86.3±21.07, respectively (Fig. 2-6).

Male-Female Courtship. Male courtship behavior toward wild-type females was not affected by ETHR silencing. In order to test male-female behavior, cantonized males were tested against *Canton-S* females. 10 male-female pairs were tested individually and courtship latency, courtship index and wing extension index were calculated.

Male-Female Courtship Index Not Affect by *Aug-21-Gal-4* Driven ETHR-RNAi. Male-female courtship did not change following ETHR silencing. RNAi(cs) males show CI±SEM of 72.83±13.5. Gal-4 only (cs), UAS only (cs) and CS males show CI of an average±SEM of 71.44±7.12, 48.36±6.36, and 52.88±4.40 respectively, towards CS females. The CI of RNAi(cs) with a $p > 0.001$ is not significantly different than its controls (Fig. 2-7 a). Wing extension index of males towards CS females was not affected after silencing ETHRs. WEI±SEM of RNAi(cs) males toward CS females is 13.50±1.96. WEI±SEM of Gal-4 only (cs), UAS only (cs) and CS are 16.90±2.05, 13.39±1.89, and 18.75±3.64 respectively. At $p > 0.1$ WEI±SEM of RNAi(cs) is not significantly different than its controls (Fig. 2-7 b).

ETHR silencing did not affect courtship latency of males toward CS females. RNAi(cs) males show average courtship latency±SEM of 21.30±6.31 towards CS females. Gal-4 only (cs), UAS only (cs) and CS show courtship latency±SEM of 15.00±2.37, 75.00±26.47 and 61.10±15.38 seconds, respectively. At $p > 0.05$, courtship latency of RNAi(cs) males was not significantly different than its control (Fig. 2-7 c).

Male Mating Behavior Not Affect by *Aug-21-Gal-4* Driven ETHR-RNAi. Male mating behavior was not altered by ETHR silencing. Mating behavior was assessed by calculating mating latency, the time from the start of courtship behavior to mating. 10 males were tested individually against CS females and mating latency was calculated for pairs that mated within 10 mins. RNAi(cs) males show an average mating latency±SEM of 280.00±31 secs. Gal-4 only (cs), UAS only (cs) and CS males show average mating latencies (in seconds) ± SEM of 282.03±52.51, 00185.26±42.05, and 343.40±52.29, respectively. Mating latencies of RNAi(cs) males are not significantly different than controls at $p > 0.1$ (Fig. 2-8).

Female Courtship Behavior. ETHR silencing does not alter female courtship behavior. Female courtship behavior was determined by testing 10 females against 10 CS males individually and courtship latency, courtship index and wing extension index of CS males towards test females was recorded.

Courtship Index of Wild-type Males Toward *Aug-21-Gal-4* Driven ETHR-RNAi Females Not Affect. Courtship index of CS males towards females was not affected after ETHR silencing. At $p > 0.1$, the average CI±SEM of CS male towards RNAi females (35.95±8.81) was not significantly different than Gal-4 only (cs), UAS only (cs) or CS control females (55.15±4.52, 29.38±6.57 and 52.88±4.04, respectively) (Fig. 2-9 a). WEI of CS males towards females was not affected after ETHR silencing. At $p > 0.01$, average WEI±SEM of CS males towards RNAi females (7.89±4.23) was not significantly

different than Gal-4 only (cs), UAS only (cs) or CS control females (19.68±2.58, 6.74±1.57, 18.75±3.64, respectively) (Fig. 2-9 b).

Courtship latency of CS males towards ETHR silenced (RNAi(cs)) females at $p > 0.1$ was not significantly different than courtship latencies towards Gal-4 only (cs), UAS only (cs) or CS females. The average courtship latency for RNAi females was 100.00±19.35 secs. as compared to Gal-4 only (cs), UAS only (cs) or CS females which was 70.10±16.07, 161.2±43.28 and 61.10±15.38 seconds, respectively (Fig. 2-9 c).

Female Mating Behavior Not Affect by *Aug-21-Gal-4* Driven ETHR-RNAi. Female mating behavior was not altered by ETHR silencing. Female mating behavior was estimated by calculating female mating latency against CS males. Mating latency for females was calculated similar to male mating latency. *Aug-21-gal-4/+* (cs) females show an average mating latency in secs. ±SEM of 375.43±28.34, *symUAS-ETHR-RNAi/+* (cs) females show 260.13±52.04 and ETHR silenced (*Aug-21-Gal-4/ symUAS-ETHR-RNAi*) females show an average mating latency ±SEM of 256.38±64.18. These latencies with p-value > 0.1 are significantly not different than CS females (343.40±52.29) (Fig. 2-10).

Aggression behavior of ETHR silenced males was not affected. Aggression behavior was determined by counting number of lunges of ETHR-RNAi males towards control flies. ETHR-RNAi flies were equally aggressive as control males (data not shown).

Fecundity Not Affect by *Aug-21-Gal-4* Driven ETHR-RNAi. Fecundity is not affected by ETHR silencing. Fecundity was measured as number of eggs produced per female per day. Average number of eggs laid per female per day by RNAi(cs) females showed an average±SEM of 28.89±0.51 eggs per day per female. This is not significantly different than Gal-4 only (cs), UAS only (cs) and CS females (28.68±0.77, 26.70±1.53 and, 22.43±0.42 respectively) (Fig. 2-11).

DISCUSSION

Behavioral analysis of ETHR silenced males revealed a significant increase in male-male courtship. ETHR silenced males court about 25-30% more to wild-type males than control males to wild-type males or wild-type males to wild-type males. This was indicated by courtship and wing extension indices of test males towards wild-type males. Increased male-male courtship toward wild-type males after ETHR silencing indicates a functional role for ETHRs in adult *Drosophila* behavior. This change in male behavior as a result of ETHR silencing could be attributed to general hyperactivity due to genetic manipulation. If silenced males are hyperactive, they would encounter other male/female more often and hence an elevated courtship index towards both females and males would be observed. In order to check if increased male-male courtship arises due to hyperactivity, ETHR silenced males were tested against wild-type virgin females. Courtship latency, courtship index and wing extension index were recorded and compared to wild-type male-female behavior. If increased male-male courtship in ETHR silenced males is due to hyperactivity, then decreased courtship latency, and increased CI

and WEI are expected. The results demonstrated that ETHR silenced male behavior towards virgin wild-type females is comparable to control male behavior towards wild-type females. There was no significant difference in CI or WEI. Although courtship latency gave a low p-value, this behavior is attributed to the Gal-4 only control background effect. This indicates that males behaved normally toward other females, but abnormally toward other males. In other words, males failed to distinguish between males and females.

Surprisingly, courtship indices of RNAi males depend on the genetic background; i.e., RNAi(cs) toward WIII8 or RNAi(w) toward CS, or other RNAi males from same background, RNAi(cs) toward RNAi(cs) or RNAi(w) toward RNAi(w), are not as elevated as toward their respective wild-type controls, RNAi(cs) toward CS or RNAi(w) toward WIII8. It is interesting to observe that RNAi males court at higher rates their respective wild-type males. In *Drosophila,* males identify other flies using specific sensory cues. Flies use visual, olfactory and gustatory inputs to identify the correct mate (Krstic et al. 2009). Courtship bias of RNAi males toward males with same genetic background indicates differences in the cuticular hydrocarbon composition of flies with different genetic background. If this is true, it also indicates that ETHR silenced flies have intact sensory ability of identifying other flies. This is an interesting result and requires more experiments for proper interpretation.

In nature, *Drosophila* males distinguish between males and females with the help of unique cuticular hydrocarbon profiles. 11-*cis*-vaccenyl acetate (cVA) is the only male

specific pheromone known in *Drosophila* to date. This volatile chemical works as an olfactory cue, which is detected with the help of olfactory receptors (ORs) OR67d and OR65a present on *Drosophila* antennal lobes (Davis 2007). Since ETHR silenced males fail to recognize other males, most likely this is due either to inability to sense male-specific smell or failure to process that smell correctly, or both. Checking levels of these receptors along with other ORs and GRs, which are known to play a role in *Drosophila* male courtship behavior, might give some insight about the cause of increased male-male courtship after ETHR silencing. (Ref. Chapter 3, Illumina sequencing was done on *Drosophila* male heads)

I observed that in the adult stage, *Aug-21-Gal-4* drives expression in many CNS neurons, including giant fiber and EH neurons. In order to determine whether these elements influence male-male courtship, various Gal-4 lines were used and courtship indices were determined against *W1118* males. The pan neuronal driver *sym-UAS-ETHR-RNAi/elav-Gal-4; tub-Gal-80^{ts}/+* was used to test if neurons are involved. Courtship behavior of *sym-UAS-ETHR-RNAi/elav-Gal-4; tub-Gal-80^{ts}/+* males was determined by testing them against *W1118* males. ETHRs are involved in a critical process in immature stages of *Drosophila* and reduction in receptor level using RNAi indicates lethal effects at the first larval stage. *sym-UAS-ETHR-RNAi/elav-Gal-4;Tub-Gal-80/+* flies were used to drive RNAi only in adult stage. *sym-UAS-ETHR-RNAi/elav-Gal-4;Tub-Gal-80/+* flies were raised at 18ºC until pupal ecdysis and 24-72 hrs later the flies were raised at 30ºC until 4 days after eclosion, when the courtship behavior of males was tested toward

W1118 males. *sym-UAS-ETHR-RNAi/elav-Gal-4;Tub-Gal-80/+* males show significantly higher courtship index towards wild-type (*W1118*) males than its controls, *sym-UAS-ETHR-RNAi/+;Tub-Gal-80/+*and *W1118* males raised at same conditions. The CI and WEI are not as high as compared to the *Aug-Gal-4* driver this can be attributed to the fact that the *elav-Gal-4* is a weak driver (Pramatarova, Ochalski et al. 2006). This shows that CNS neurons are involved in male-male courtship regulation downstream of ETHR-RNAi. In order to silence ETHRs in giant fiber neurons, *A307-Gal-4/sym-UAS-ETHR-RNAi* males were tested against *W1118*. The results show that giant fiber neurons are not involved in regulation of male courtship behavior.

Since *fruitless* and *doublesex* neurons are known to be involved in male-male courtship interactions, receptors were silenced in those neurons and courtship behavior was analyzed. ETHR silencing in *fruitless* neurons led to significant elevation of the male CI. This shows that ETHRs located on *fruitless* neurons play a role in regulating male-male courtship behavior. Surprisingly, wing extension index was not as high as that observed using the *Aug21-Gal-4* line. This suggests that ETHRs in CA regulate wing extension behavior during male-male courtship. When ETHRs were silenced in *doublesex* neurons using *dsx-Gal-4* line, ETHR silenced males did not court other males significantly differently than their control males. This shows that ETHRs are either not present on the *dsx* neurons or do not play a role in male-male courtship behavior.

The CA are known to have gonadotropic influences in flies. Hence, it is important to determine if mating behavior of both males and females is affected. Mating latency is a

good indicator of mating behavior. If the ETHRs play a role in mating behavior, one would expect to observe a change in mating latency after ETHR silencing. In other words, if male mating behavior is affected due to ETHR silencing, the wild-type virgin might take longer or shorter time to accept males, leading to changes in mating latency. Similarly, if ETHR silencing has an effect on female mating behavior (indicated by female receptivity), wild-type males should take a longer or shorter time to mate with ETHR silenced females. I observed that ETHR silenced males mate equally efficiently with wild-type females. The mating latency of males towards wild-type females was not affected after ETHR silencing. With regard to female mating behavior, ETHR silenced females were equally receptive to wild-type males. The ETHR silenced female mating latency with wild-type males was not different than control females.

Since ETHRs are silenced in CA, a gland producing JH which plays a major role in egg production in females, it was hypothesized that the ETHRs may play a role in female fecundity (Riddiford 2008). However, there was no significant difference in the number of eggs laid per ETHR silenced female per day. This result indicates that ETHRs in CA do not play a role in female fecundity.

Overall, the results indicate that ETHR silencing in CA affects only male courtship behavior, whereas there is no effect on female behavior. ETHRs to date were known to play a major role in ecdysis. My results show a novel function of ETHRs in adult *Drosophila.* This is a very interesting result as it shows how genes change function after metamorphosis. Some of the hormones known to change function after

metamorphosis include juvenile hormone and ecdysone, both of which have developmental roles in immature stages and as well as later in the adult stage. Results of experiments shown here indicate a similar pattern of regulation by ETHRs in immature stages (larval and pupal stage) they play a role in ecdysis, while later in the adult stage they play a role in male courtship behavior.

It is not clear if the function of ETHRs in adult males is to regulate JH synthesis. However, since there was no effect on female fecundity, it is reasonable to conclude that ETHRs do not regulate JH related behaviors in adult females. Recent stage specific RNAseq data in *Drosophila* shows presence of ETH transcripts in both adult males and females, but presence of ETHR transcripts only in adult males. Absence of ETHR transcripts in females may explain the lack of behavioral changes in ETHR silenced adult females, but could suggest the presence of novel unidentified receptors for ETH. This is a very interesting result and is open for further investigation.

CONCLUSIONS

ETHRs are known to play a role in ecdyis behavior in the immature stages of insects. Given their critical roles in ecdysis behaviors, it is intriguing that they persist in the adult stages. Hormones like ecdysone and JH are known to play a role in the immature and adult stages. Both these hormones switch roles after metamorphosis. It is interesting to see a novel function of ETHRs in the adult stage. ETHR-RNAi in neurons and CA shows that these receptors are involved in male courtship behavior. Absence of

female behavioral change is explained by the absence of transcripts in adult females. Presence of ETH transcripts in both males and females adults and absence of receptors in adult females suggests unidentified receptors for ETH in adult females.

Courtship behavior is known to be regulated by a number of genes. ETHR-RNAi experiments help us to conclude that ETHRs have a novel function in the adult stage and are involved in inhibiting male-male courtship behavior. Increased male-male courtship after silencing ETHRs in *fruitless* neurons and CA show that ETHRs present on CA and other neurons are involved in regulating courtship behavior. The change in behavior after ETHR silencing could be a combined effect of both CA and neurons. The exact mechanisms by which ETHRs regulate downstream courtship behavior in males are not clear and require further experimentation.

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Figure 2-1. **Thoracic ETH expressing Inka cells persist in adult flies**. 5 day old adult male thorax showing tdTomato expression under ETH-Gal-4. White arrows indicate Inka cells. Inset shows one thoracic Inka cell on the trachea.

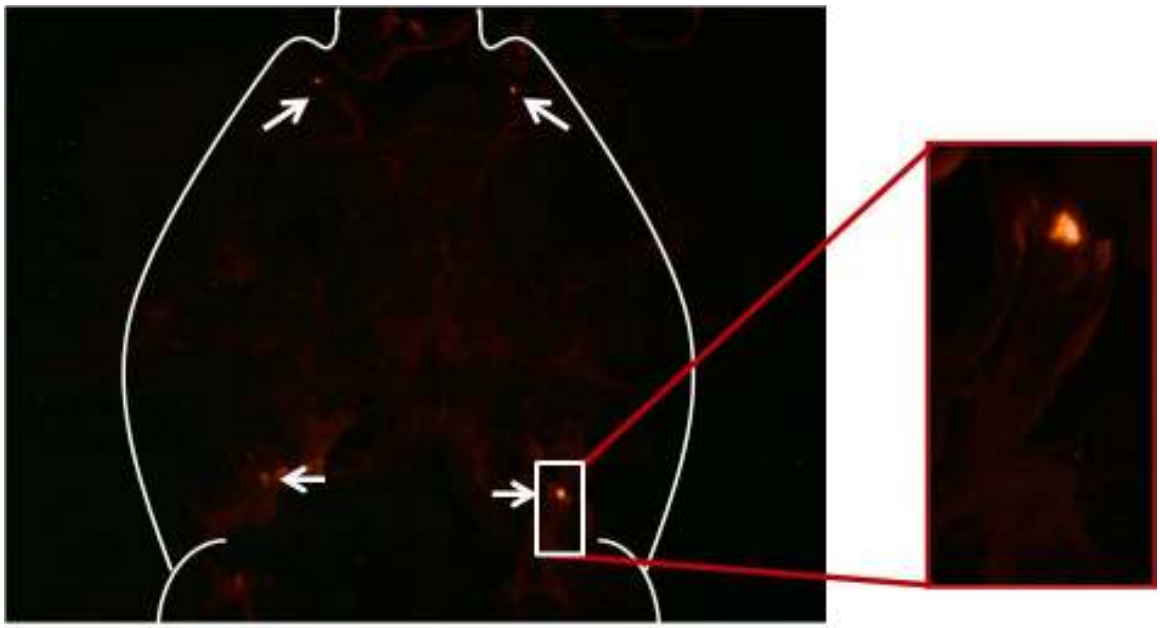
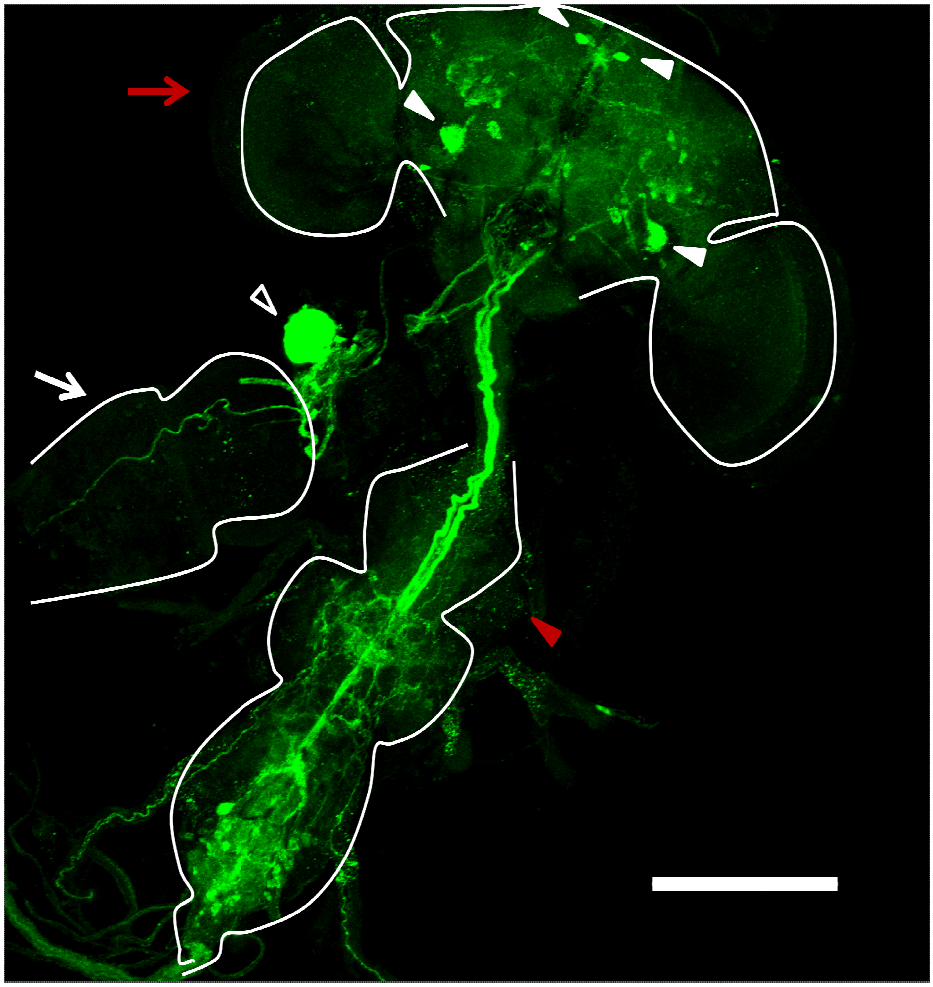


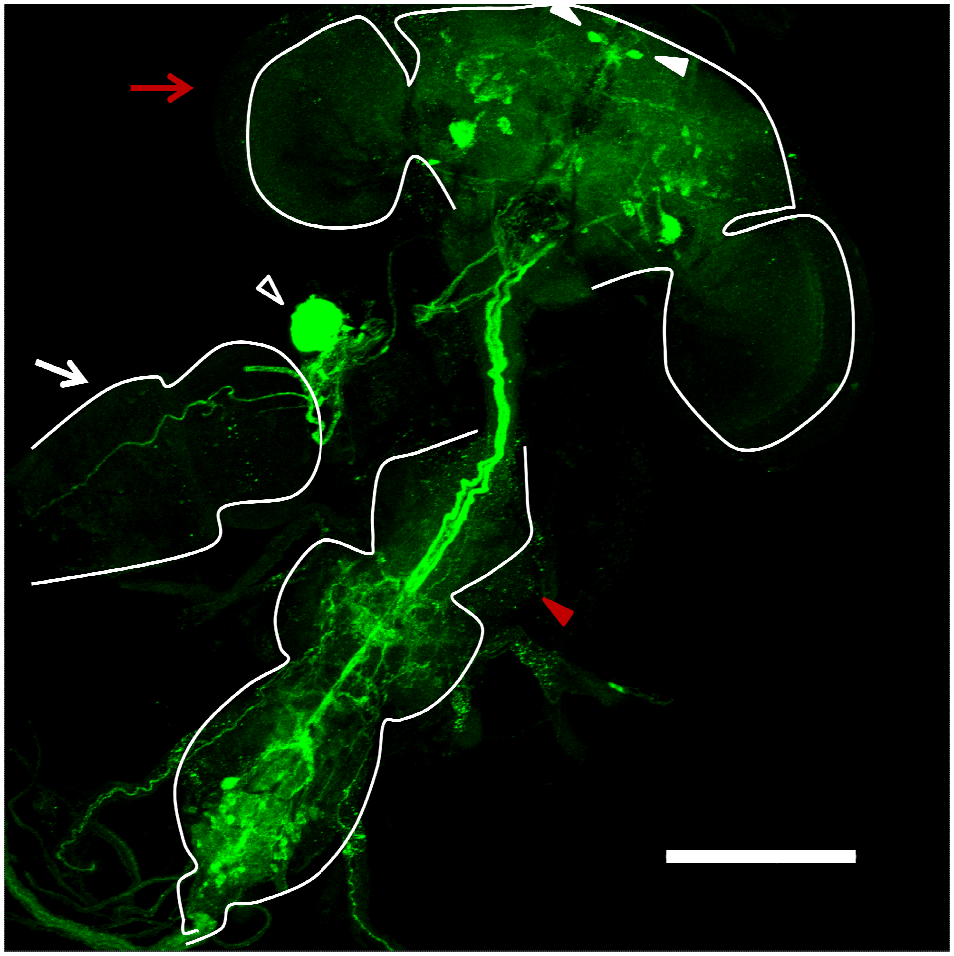
Figure. 2-1.

Figure 2-2. Immunostaining on *Aug-21-Gal-4/UAS-CD8m-GFP* 3 day old males. a) Open white arrow head indicates corpora allata, white closed arrow heads indicate neuronal cell bodies, red arrow indicates brain, white arrow indicates gut and red closed arrow head indicates thoracic and abdominal neuromeres. White bar indicates a scale of 150µm. **b)** Cell bodies of EH neurons marked with white arrow heads **c))** Cell bodies of giant fiber neurons marked with white arrow heads **d)** Neuronal projections of *Aug-21-Gal-4*similar to giant fiber projections.

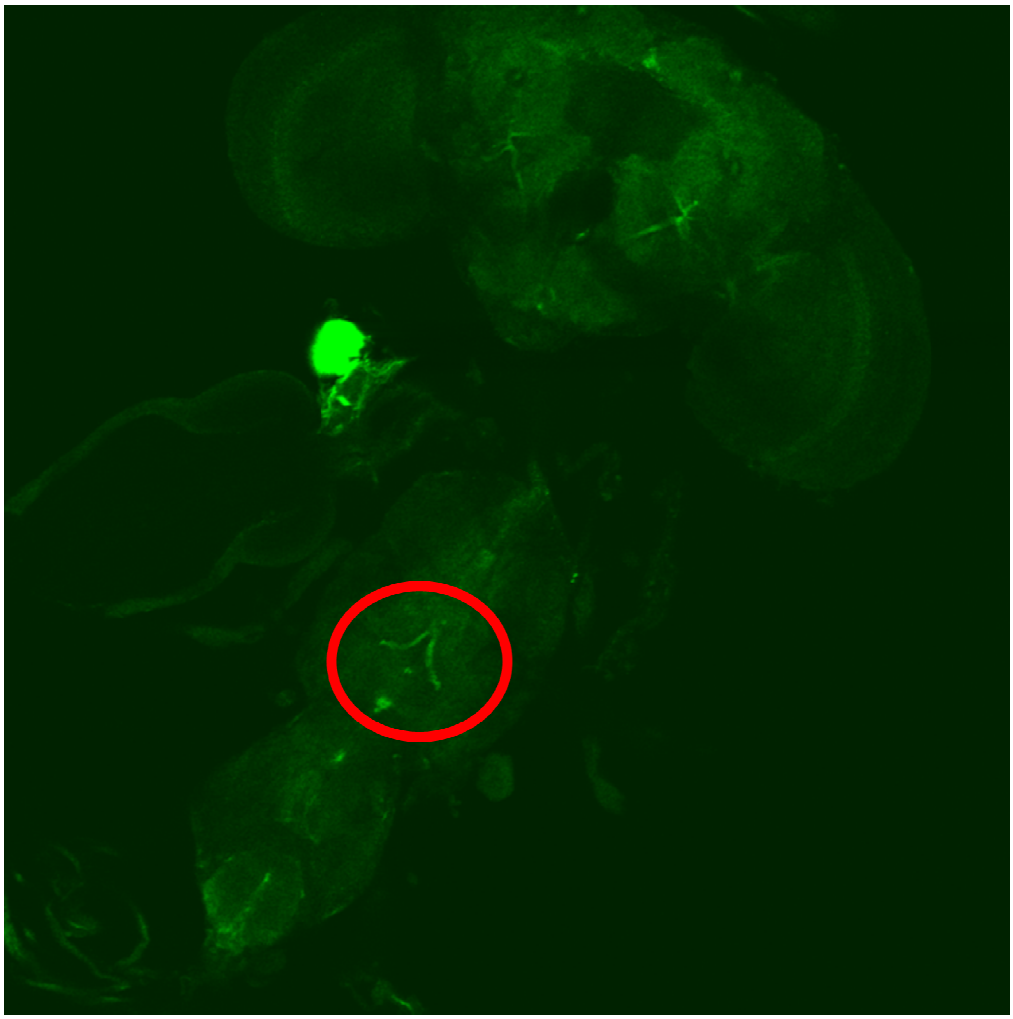
a)



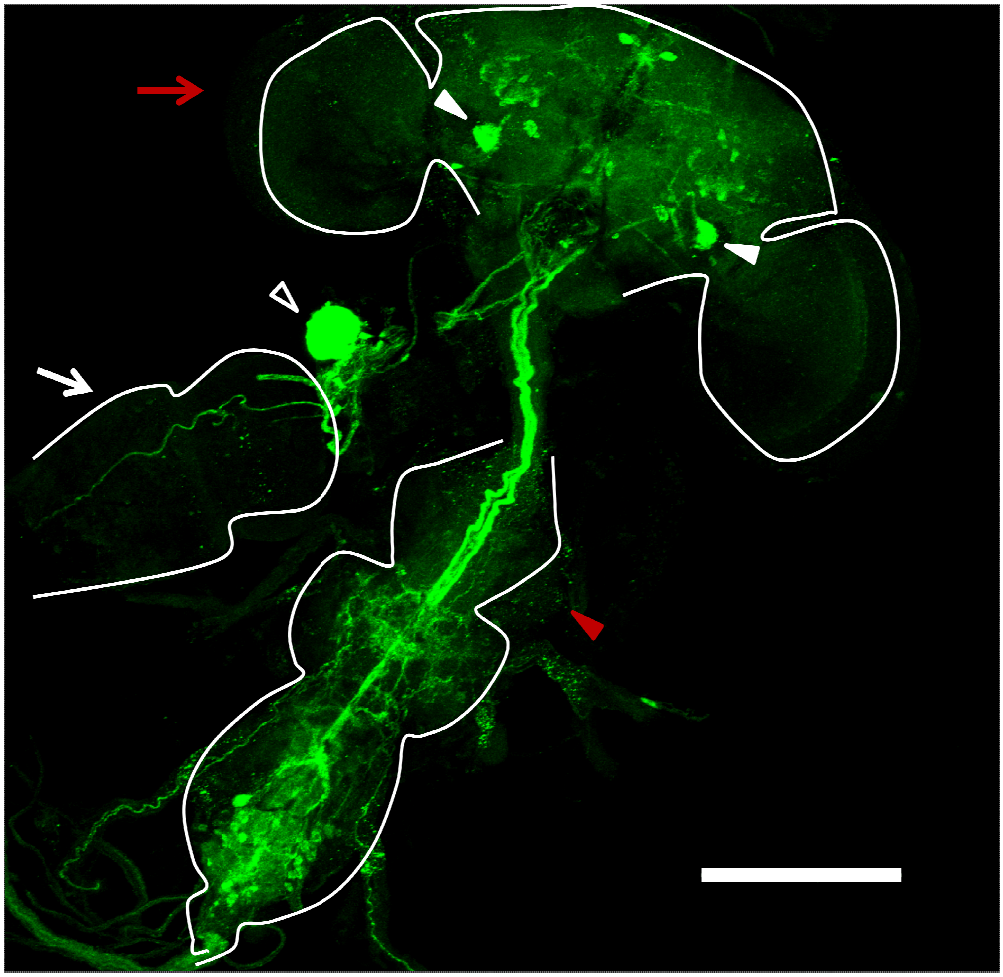
b)



c)



d)



e)

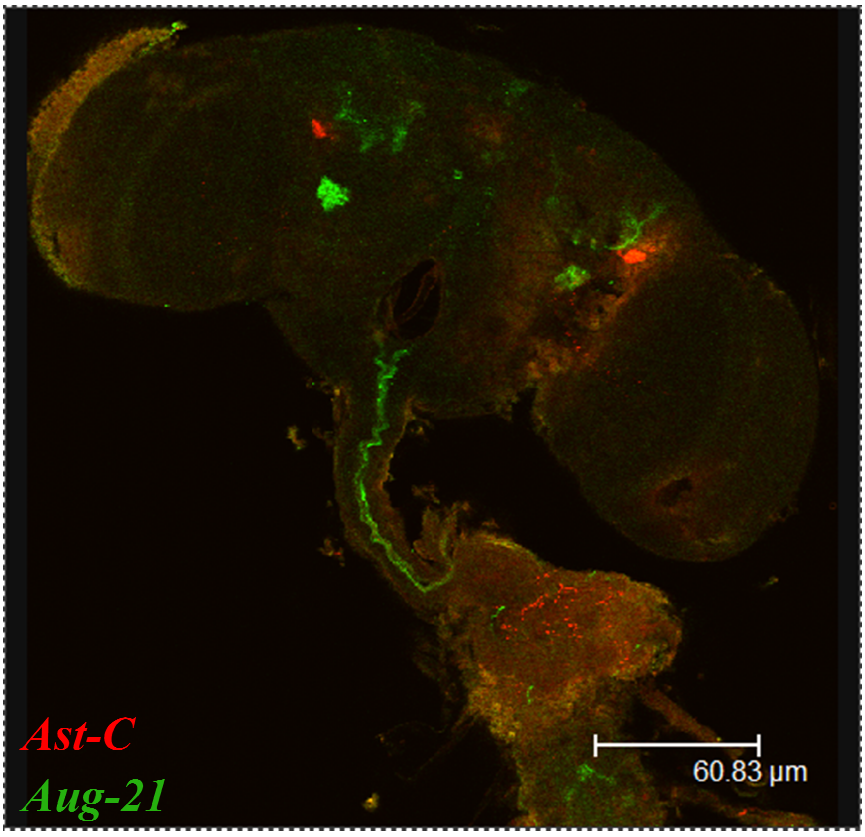
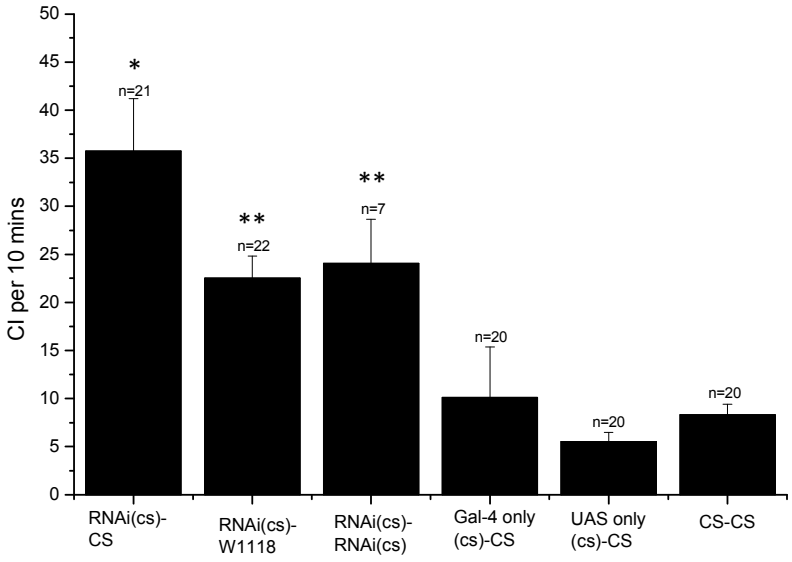


Figure 2-2.

Figure 2-3. ETHR silencing elevates male-male courtship index.

a) Courtship index of cantonized males towards other males. There was no significant difference in courtship index between Gal-4 only, UAS only and *Canton-S* (CS) controls. ETHR silenced males (RNAi(cs)) show significantly higher courtship index towards males. **b)** Courtship index of *W1118* background males towards males. There was no significant difference in courtship index between Gal-4 only, UAS only and *W1118* controls. ETHR silenced males (RNAi(w)), show significantly higher courtship index towards males. A Kruskal-Wallis analysis with a *post hoc* Mann-Whitney's test was performed. Error bars represent standard error mean (SEM). * indicates $p < 0.0001$ and ** indicates $p < 0.001$.

a)


CI per 10 mins
50
45
40
35
30
25
20
15
10
5
0
*
n=21
**
n=22
**
n=7
n=20
n=20
n=20
RNAi(cs)-CS
RNAi(cs)-W1118
RNAi(cs)-RNAi(cs)
Gal-4 only (cs)-CS
UAS only (cs)-CS
CS-CS

b)

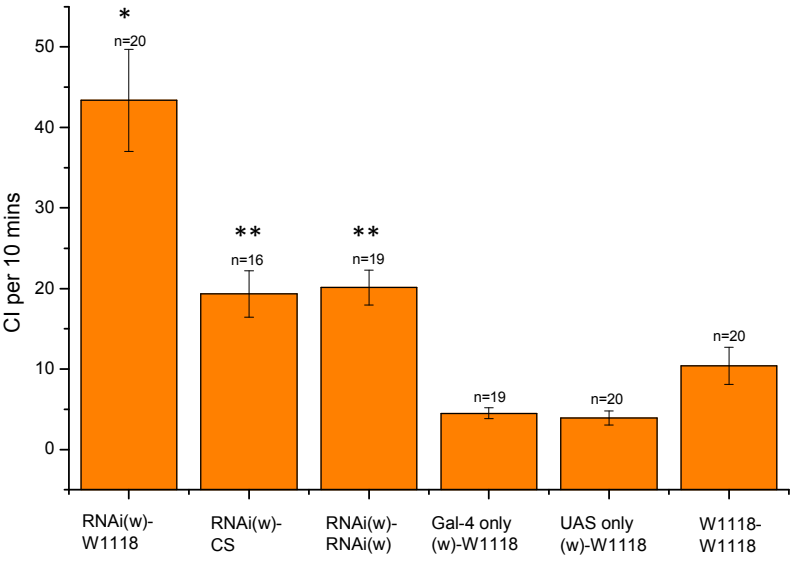

CI per 10 mins
50
40
30
20
10
0
*
n=20
**
n=16
**
n=19
n=19
n=20
n=20
RNAi(w)-W1118
RNAi(w)-CS
RNAi(w)-RNAi(w)
Gal-4 only (w)-W1118
UAS only (w)-W1118
W1118-W1118

Figure 2-3.

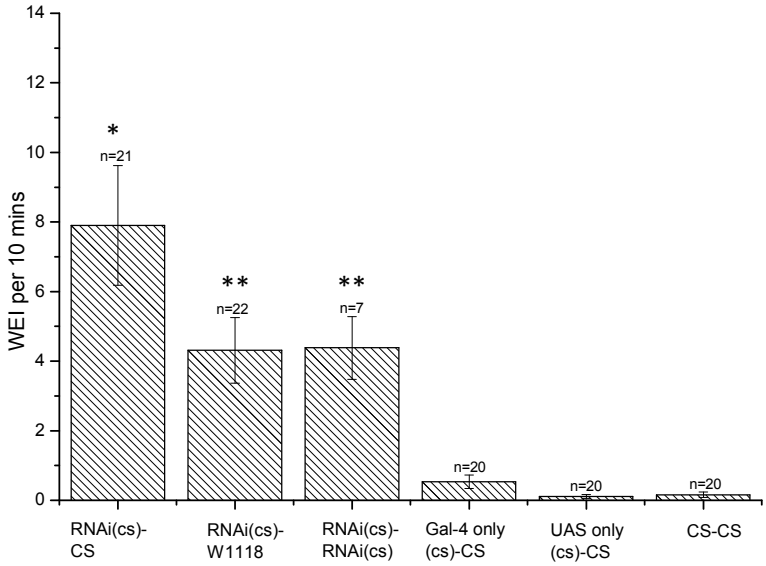
Figure 2-4. Courtship indices of males with ETHR-RNAi using various Gal-4 lines towards *W1118* males. A Kruskal-Wallis analysis with a *post hoc* Mann-Whitney's test was performed. Error bars represent standard error mean (SEM). ** indicates $p < 0.001$.

CI per 10 mins
0
5
10
15
20
25
30
35
40
n= 20
n= 14
**
n= 9
n= 6
n= 13
**
n= 6
n= 10
n= 10
n= 9
n= 13
W1118
elav-Gal-4/RNAi; tub-Gal-80ts
elav-Gal-4/+; tub-Gal-80ts
EH-Gal-4/RNAi
fuMGal-4/RNAi
dsx-Gal-4/RNAi
Ast-C-Gal-4/RNAi
A307-Gal-4/RNAi
AKH-Gal-4/RNAi
AKH-Gal-4/+
Test Genotype
W1118
Subject Genotype

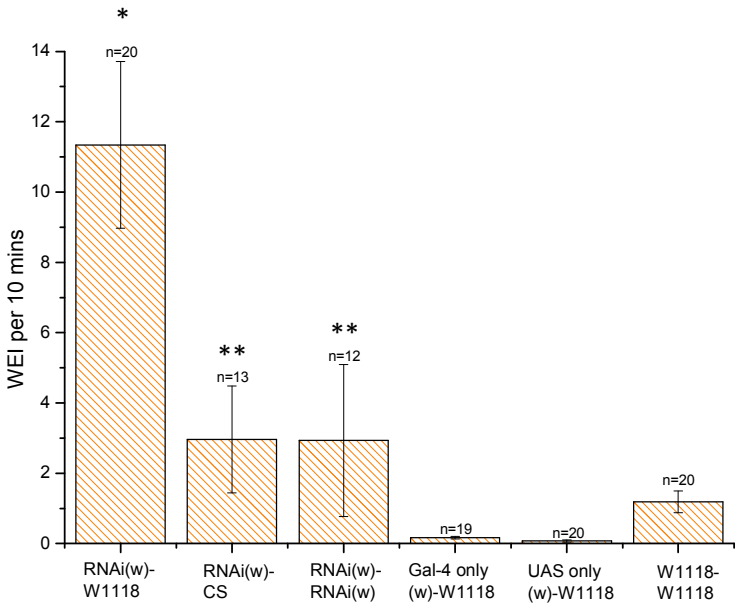
Figure 2-4.

Figure 2-5. ETHR silencing elevates male-male wing extension index. a) Wing extension index of cantonized males towards males. There was no significant difference in wing extension index between Gal-4 only, UAS only and *Canton-S* (CS) controls. ETHR silenced males (RNAi(cs)) show significantly higher WEI towards males. **b)** Wing extension index of *W1118* background males towards males. There was no significant difference in wing extension index between Gal-4 only, UAS only and *W1118* controls. ETHR silenced males (RNAi(w)), show significantly higher WEI towards males. A Kruskal-Wallis analysis with a *post hoc* Mann-Whitney's test was performed. Error bars represent standard error mean (SEM). * indicates $p < 0.0001$, ** indicates $p < 0.001$ and *** indicates $p < 0.05$.

a)


WEI per 10 mins
14
12
10
8
6
4
2
0
*
n=21
**
n=22
**
n=7
n=20
n=20
n=20
RNAi(cs)-CS
RNAi(cs)-W1118
RNAi(cs)-RNAi(cs)
Gal-4 only (cs)-CS
UAS only (cs)-CS
CS-CS

b)


WEI per 10 mins
14
12
10
8
6
4
2
0
*
n=20
**
n=13
**
n=12
n=19
n=20
n=20
RNAi(w)-W1118
RNAi(w)-CS
RNAi(w)-RNAi(w)
Gal-4 only (w)-W1118
UAS only (w)-W1118
W1118-W1118

c)

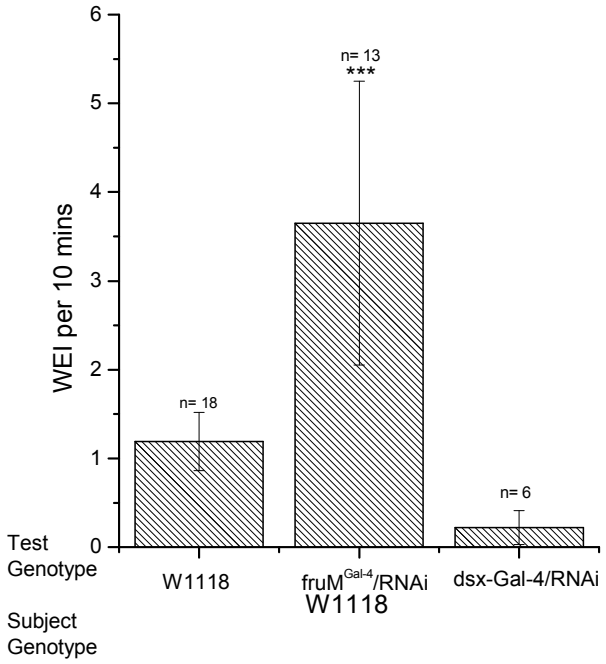

6
5
4
3
2
1
0
WEI per 10 mins
n= 13
***
n= 18
n= 6
Test Genotype
W1118
fruM^Gal-4/RNAi
dsx-Gal-4/RNAi
Subject Genotype
W1118

Figure 2-5.

Figure 2-6. Male courtship latency is not affected by ETHR silencing. Courtship latency of cantonized males towards *Canton-S* (CS) males. There was no significant difference in the courtship latency of ETHR silenced males towards CS males. A Kruskal-Wallis analysis with a *post hoc* Mann-Whitney's test was performed. Error bars represent standard error mean (SEM).

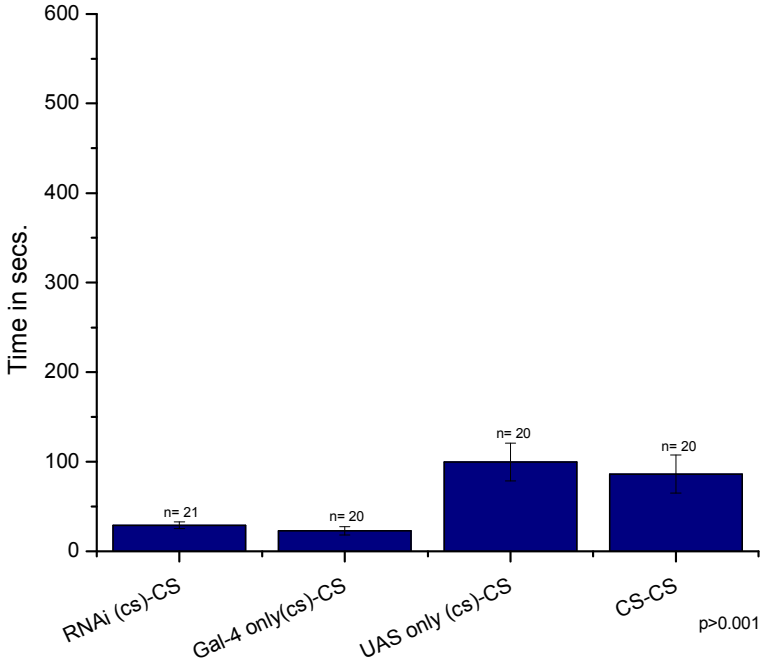
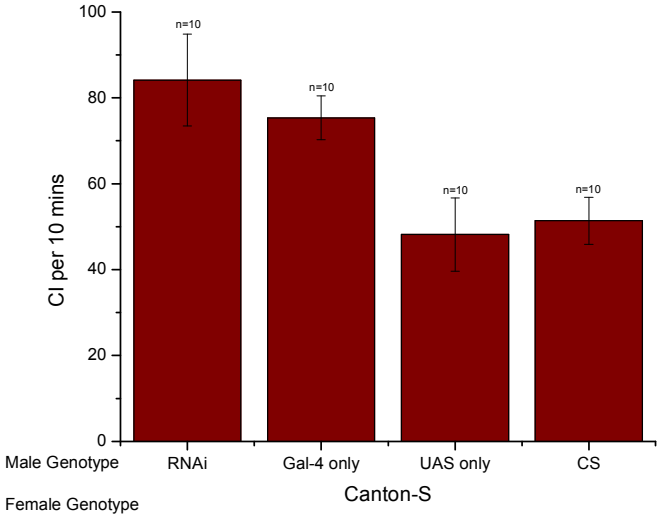

600
500
400
300
200
100
0
Time in secs.
n= 21
n= 20
n= 20
n= 20
RNAi (cs)-CS
Gal-4 only(cs)-CS
UAS only (cs)-CS
CS-CS
p>0.001

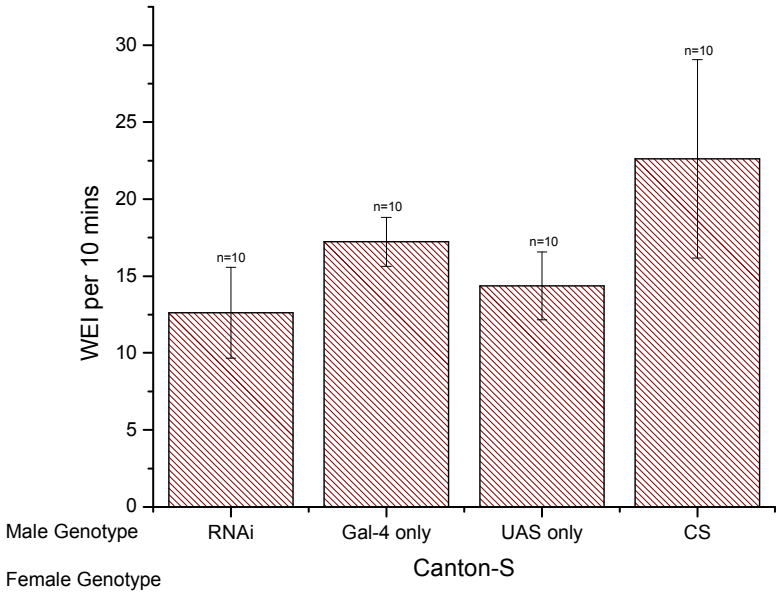
Figure 2-6.

Figure 2-7. ETHR silencing does not affect male courtship behavior towards *Canton-S* (CS) females. a) Courtship index of cantonized males towards CS females. There is no significant difference in courtship index of RNAi males towards CS females. **b)** Wing extension index of cantonized males towards CS females. There is no significant difference in wing extension index of RNAi males towards CS females. **c)** Courtship latency of cantonized males towards CS females. There was no significant difference in the courtship latency of RNAi males towards CS females. A Kruskal-Wallis analysis was performed. Error bars represent SEM.

a)


100
80
60
40
20
0
CI per 10 mins
n=10
n=10
n=10
n=10
Male Genotype
RNAi
Gal-4 only
UAS only
CS
Female Genotype
Canton-S

b)


30
25
20
15
10
5
0
WEI per 10 mins
n=10
n=10
n=10
n=10
Male Genotype
RNAi
Gal-4 only
UAS only
CS
Female Genotype
Canton-S

c)

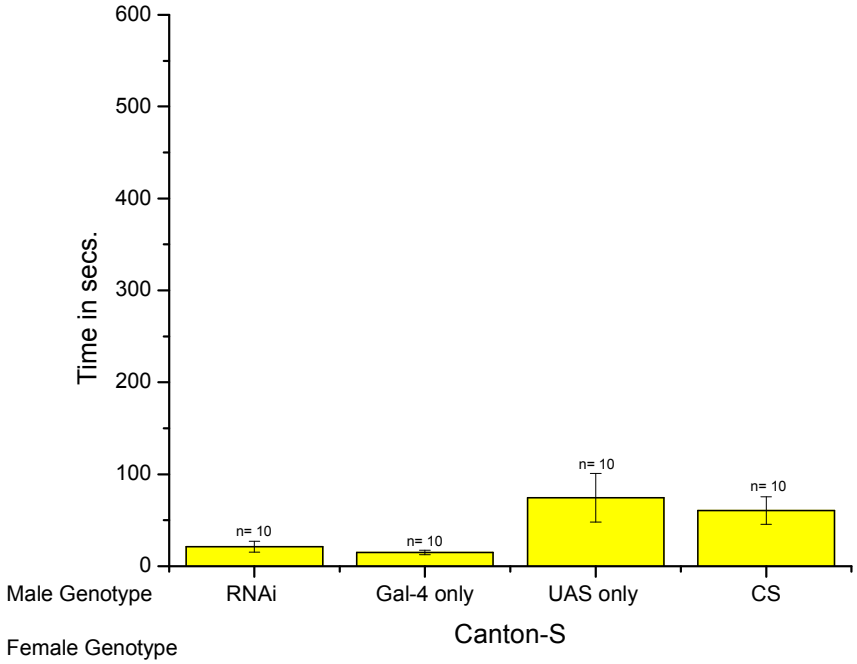

600
500
400
300
200
100
0
Time in secs.
n= 10
n= 10
n= 10
n= 10
Male Genotype
RNAi
Gal-4 only
UAS only
CS
Female Genotype
Canton-S

Figure 2-7.

Figure 2-8. ETHR silencing does not affect male mating behavior. RNAi males show no significant difference in mating latency towards CS females. A Kruskal-Wallis analysis was performed. Error bars represent SEM.

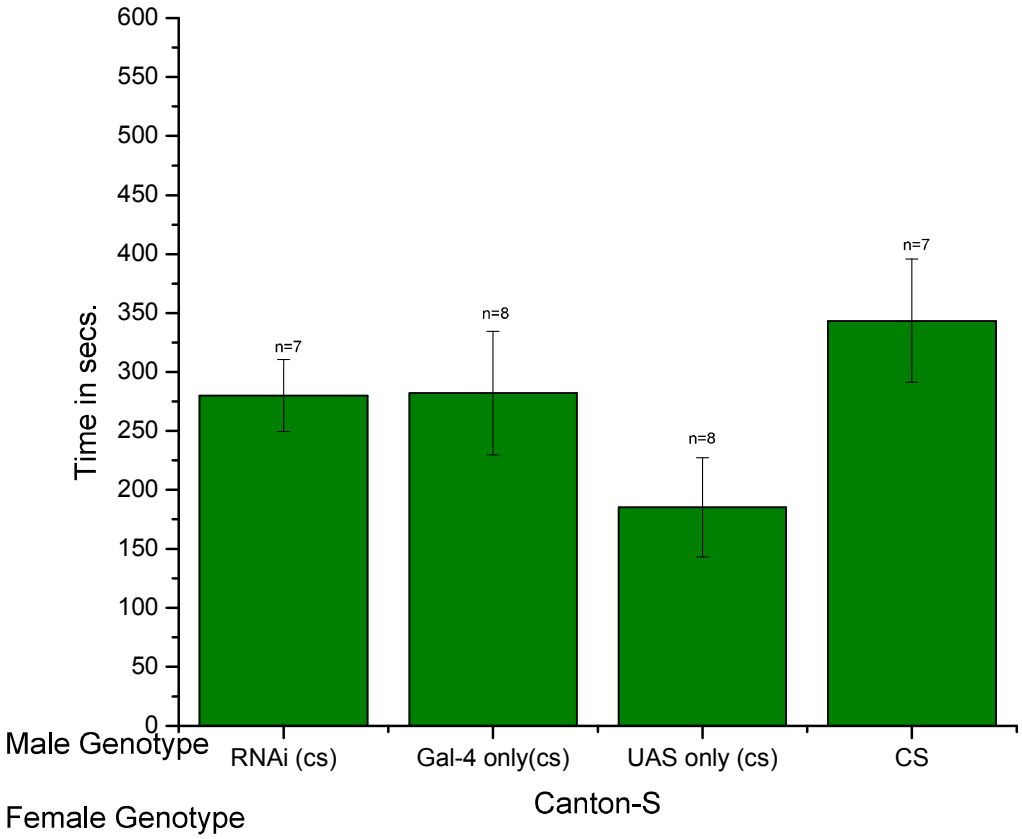
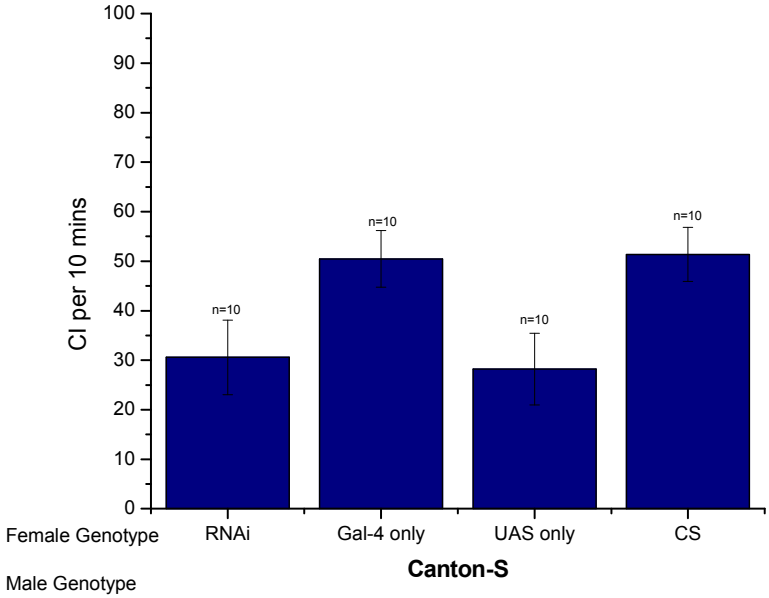

600
550
500
450
400
350
300
250
200
150
100
50
0
Time in secs.
n=7
n=8
n=8
n=7
Male Genotype
RNAi (cs)
Gal-4 only(cs)
UAS only (cs)
CS
Female Genotype
Canton-S

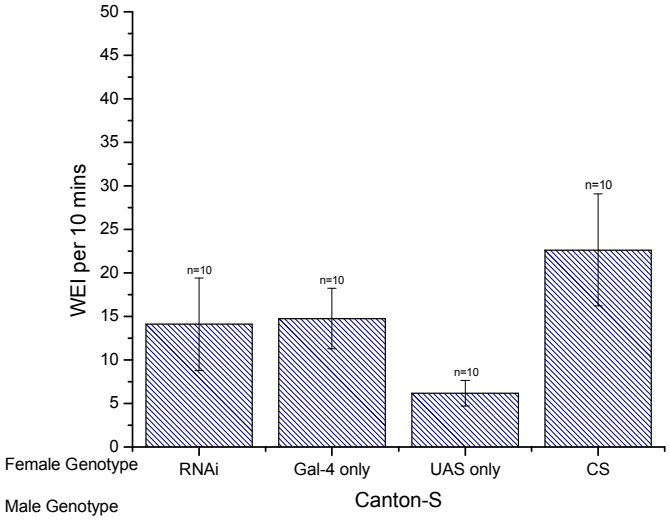
Figure 2-8.

Figure 2-9. Courtship index of wild-type males toward ETHR silenced females not affected. Effect of ETHR silencing on female courtship behavior against *Canton-S* (CS) males. **a)** Courtship index of CS males towards cantonized test females. There is no significant difference in courtship index of CS males towards RNAi females. **b)** Wing extension index of CS males towards cantonized test females. There is no significant difference in wing extension index of CS males towards RNAi females. **c)** Courtship latency of CS males towards cantonized test females. There was no significant difference in the courtship latency of CS males towards RNAi females. A Kruskal-Wallis analysis was performed. Error bars represent SEM.

a)


CI per 10 mins
100
90
80
70
60
50
40
30
20
10
0
n=10
n=10
n=10
n=10
Female Genotype
RNAi
Gal-4 only
UAS only
CS
Male Genotype
Canton-S

b)


WEI per 10 mins
50
45
40
35
30
25
20
15
10
5
0
n=10
n=10
n=10
n=10
Female Genotype
RNAi
Gal-4 only
UAS only
CS
Male Genotype
Canton-S

c)

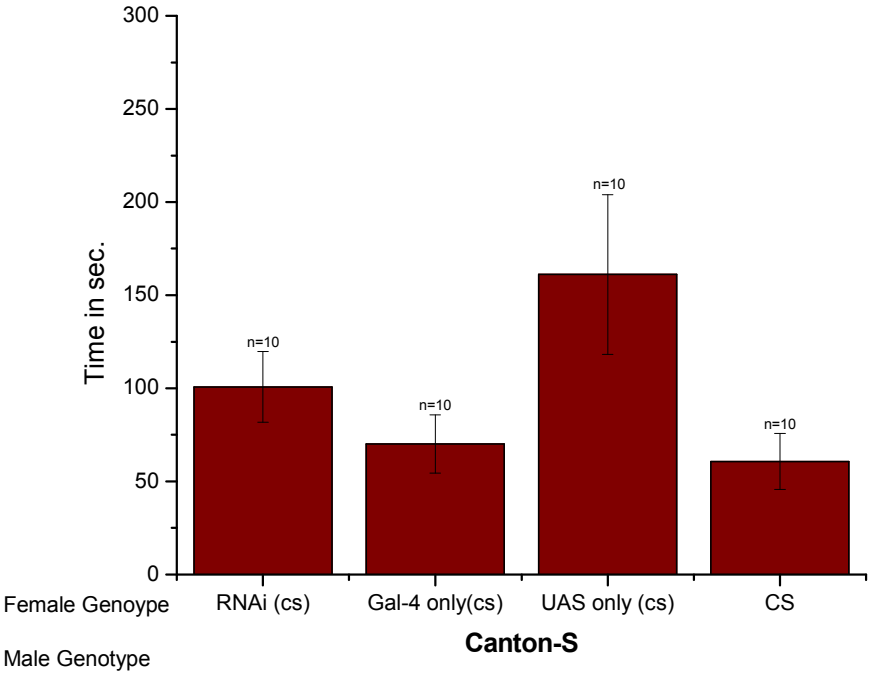

300
250
200
150
100
50
0
Time in sec.
n=10
n=10
n=10
n=10
Female Genoype
RNAi (cs)
Gal-4 only(cs)
UAS only (cs)
CS
Male Genotype
Canton-S

Figure 2-9.

Figure 2-10. ETHR silencing does not affect female mating behavior. RNAi females show no significant difference in mating latency towards CS males. A Kruskal-Wallis analysis was performed. Error bars represent SEM.

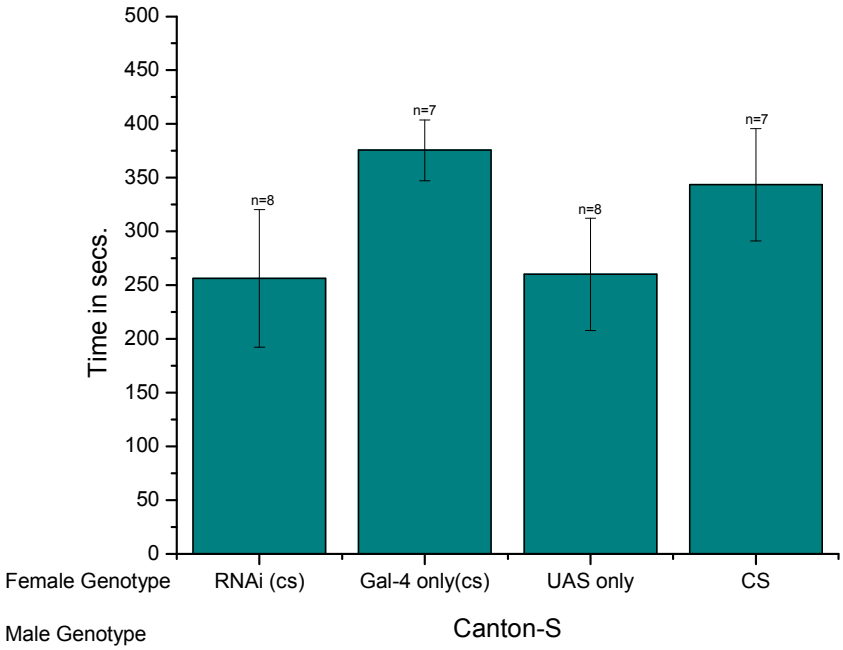

500
450
400
350
300
250
200
150
100
50
0
Time in secs.
n=8
n=7
n=8
n=7
Female Genotype
RNAi (cs)
Gal-4 only(cs)
UAS only
CS
Male Genotype
Canton-S

Figure 2-10.

Figure 2-11. ETHR silencing does not affect female fecundity. There was no significant difference in number of eggs laid per day per female by RNAi females. A Kruskal-Wallis analysis was performed. Error bars represent SEM.

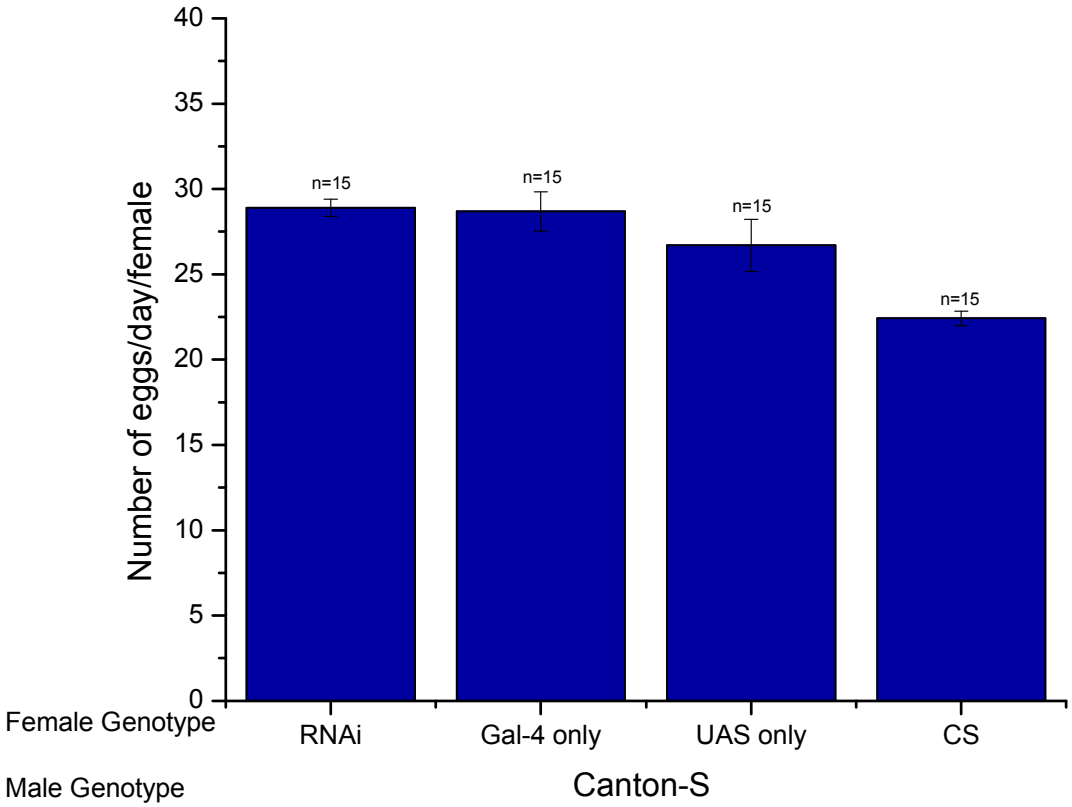

Number of eggs/day/female
40
35
30
25
20
15
10
5
0
n=15
n=15
n=15
n=15
Female Genotype
RNAi
Gal-4 only
UAS only
CS
Male Genotype
Canton-S

Figure 2-11.

CHAPTER III

Transcriptome Analysis of Ecdysis Triggering Hormone Receptor Silenced Adult Male *Drosophila melanogaster*.

ABSTRACT

Ecdysis triggering hormone regulates ecdysis behavior in insects via ETHRs to activate a peptide signaling cascade in the CNS. Presence of ETHRs in corpora allata (CA) was reported recently in the silkworm, *Bombyx mori*. Chapter 2 shows silencing of ETHRs using *Aug-21-Gal-4,* a corpora allata driver, elevates male-male courtship behavior. This study focuses on patterns of altered gene expression resulting from ETHR-RNAi. RNAseq analysis was performed on three samples: CA, head, and whole fly. This is the first study in which transcriptome analysis has been done on CA. Genes previously considered to be expressed exclusively in male accessory glands are found in CA. ETHR-RNAi also alters expression of JH related genes, suggesting changes in JH levels may underly increased male-male courtship.

ETHR-RNAi resulted in differential expression of 2901 genes. ~95 histone genes were down-regulated, suggesting changes in chromatin organization. Differentially expressed genes from the CA library were clustered on the chromosome, providing further evidence of changes in chromatin organization. Differentially expressed genes included ~68 transcription factors. *Doublesex* (*dsx),* one of the sex determination genes, is down-regulated in ETHR silenced flies. Genes such as *acj6, Dscam3* and *Dad,* which are involved in axon guidance and male courtship behavior were differentially expressed. The sensory system plays important roles in regulating male courtship behavior and ETHR-RNAi was shown to regulate a number of genes associated with sensory functions. Juvenile hormone-related genes, including juvenile hormone acid methyl

transferase (*jhamt),* Daughters against dpp *(Dad), broad (br)* and juvenile hormone induced protein-1 *(jhI-1)* were differentially expressed after ETHR-RNAi. Up-regulation of *jhamt* and *jhI-1,* and down-regulation of *broad* and *Dad* indicates that ETHR-RNAi increases JH levels in adult males.

Based on the analysis of differentially expressed genes, I hypothesize that ETH has an allatostatic function in *Drosophila* males; disrupting ETH signaling as a result of ETHR-RNAi would therefore cause an increase in JH production. Differential expression of JH related genes and chromatin organization genes as a result of ETHR-RNAi led to formulation of a model for regulation of male courtship behavior by ETHRs. ETHRs regulate JH levels and chromatin organization, which affect sensory system genes and male courtship behavior. It is also possible that increased levels of JH cause chromatin reorganization or alternatively chromatin organization might be affecting JH levels. Overall, this model could explain the male-male courtship phenotype observed after ETHR-RNAi. In addition, RNAseq analysis led to discovery of a new exon in the sex determination gene *doublesex* in *Drosophila* males.

INTRODUCTION

Drosophila is widely used as a model organism to investigate genes involved in complex processes like learning and memory, circadian rhythm, courtship and aggression (Siegel et al. 1979; Belvin et al. 1997; Levine et al. 2002). I have shown in the previous chapter that ETHRs are involved in regulation of adult male courtship behavior. A genomic approach was taken in order to investigate the transcriptional and post-

transcriptional mechanisms functioning in this behavioral change. Recently, genomic approaches have been used for identification of candidate genes involved in regulating many behaviors (Edwards et al. 2006; Carney 2007; Bonizzoni et al. 2011; Ellis et al. 2011).

ETHRs are G-protein coupled receptors, which activate the Gαq pathway to mobilize intracellular calcium. ETH signaling ultimately results in activation of centrally patterned behaviors that promote shedding of exocuticle. ETH is produced by Inka cells of the epitracheal gland. These cells persist in the adult stage, suggesting functional roles for ETH signaling in adults. ETHRs occur as two splice variants, ETHR-A and ETHR-B, which have different 3' exons. Most ETHR-A neurons are peptidergic and their specific roles in ecdysis scheduling have been determined. Recently, ETHRs were reported in the corpora allata of the 4^{th} and 5^{th} instar silkworm, *Bombyx mori* (Yamanaka et al. 2008). ETHR-RNAi using the *Aug21-Gal-4* driver, which specifies expression in adult CA and a subset of central neurons results in elevated male-male courtship (see Chapter II).

Courtship is one of many well-described innate behaviors in *Drosophila*. Various genes are implicated in regulation of male-male courtship behavior. Among these are the transcription factors *fruitless (fru), doublesex (dsx),* gustatory and olfactory receptors, the *ecdysone receptor (EcR*), the *white* gene (*w)* and ion channels like *cacophony* (*cac)* (Zhang et al. 1995; Demir et al. 2005; Villella et al. 2008; Brigitte et al. 2011; Ganter et al. 2011). Studies also have focused on pheromones and neuronal circuitry involved in regulation of male-male courtship.

This study focuses on molecular mechanisms involved in elevation of male-male courtship resulting from ETHR-RNAi. To accomplish this, I performed RNAseq analysis on three samples: corpora allata, heads and whole flies. Whereas all transcriptome studies so far have focused on one tissue, making it difficult to see the effect on genes in individual tissues, these three samples were chosen to examine tissue specific changes in gene expression.

MATERIALS AND METHODS

Insect Rearing. *Drosophila melanogaster* were reared on regular cornmeal medium at 25ºC under 12:12 light: dark cycle. *symUAS-ETHR-RNAi* flies were obtained from our laboratory stock, wCS (cantonized *W1118*) flies were gifted by Dr. Dahanukar, *Aug21-Gal-4/cyo* flies were gifted by Dr. Korge. *Aug21-Gal-4/cyo* and *symUAS-ETHR-RNAi* flies were cantonized by back-crossing flies to wCS flies for 5 generations. *Aug21-Gal-4/symUAS-ETHR-RNAi* flies were obtained by crossing *Aug21-Gal-4/cyo* flies with *symUAS-ETHR-RNAi. Aug21-Gal-4/+* were obtained by crossing *Aug21-Gal-4/cyo* flies with *CS/W1118* and were used as Gal-4 only controls. *symUAS-ETHR-RNAi* flies were crossed with *CS/W1118* and *symUAS-ETHR-RNAi/+* flies were used as UAS only controls. All male flies were collected within 12 hrs after eclosion under CO_2 anesthesia. Males were individually aged after collection in 12 X 75mm pyrex glass culture tubes (Corning, NY, United States) with 1.5cm food at the bottom.

Sample Collection and Processing for Illumina Sequencing.

Whole Flies. Ten 3-5 day old socially isolated males of each genotype were collected by snap freezing flies into liquid nitrogen at Zeitgeber time (ZT) 7, the same time that behaviors were tested. Total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and followed by DNase treatment with Turbo DNA-free (Ambion). For further purification, RNA was column cleaned using RNeasy MinElute Cleanup Kit (Qiagen). Poly-A containing mRNA was isolated from total RNA using oligo-dT magnetic beads (Dynabeads, Invitrogen). Further, RNA fragmentation was done using 5X fragmentation buffer and fragmented RNA was purified by glycogen and ethanol precipitation. In order to check RNA integrity, samples were subjected to Agilent 2100 Bioanalyzer, located at Institute for Integrative Genome Biology, University of California, Riverside (IIGB, UCR). First strand cDNA was synthesized using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). Second strand cDNA was synthesized using RNaseH and DNA polymerase I (NEB) and this double stranded cDNA (ds-cDNA) was purified using the Qiaquick PCR purification kit (Qiagen). NEBNext DNA sample prep master mix set 1 was used for further processing and protocols were followed according to manufacturer's instructions. End repair to make blunt ds-cDNA was performed with NEBNext end repair enzyme mix for 30 mins at 20ºC. A single adenine base was added to the blunt ds-cDNA using Klenow fragment (3'→ 5' exo-nuclease), and was ligated to the locked nucleic acid (LNA) adaptors with barcodes using T4 DNA ligase (NEB). Further, ~250 bps size selection was

done on a 2% agarose E-gel (Invitrogen), followed by PCR amplification of ligated DNA using Phusion high-fidelity enzyme (Fermentas). Samples were subjected to single-end sequencing on an Illumina Genome Analyzer II platform (Hiseq2000) at the IIGB, UCR. Libraries were run at a concentration of 2.25 pM using 50 cycles. A total of 4 samples were multiplexed together in one lane.

Fly Heads. 3-5 day old 50 adult naïve male heads were collected by snap freezing flies into liquid nitrogen at ZT 7. All head samples were processed similar to whole fly samples with size selection of 300 bps. Samples were submitted to the IIGB, UCR and were subjected to single-end sequencing on the HiSeq2000 (Illumina). Libraries were run at a concentration of 1.375 pM using 100 cycles. A total of 4 samples were multiplexed together in one lane.

Fly Corpora allata. Corpora allata along with adjacent tissue, including corpora cardiac and gut were dissected from ten, 3-5 day old naïve adult males at ZT 7. The tissue was stored in RNase free tubes kept on dry ice. Corpora allata RNA samples were processed using the Ovation RNA-Seq System (NuGen) following the manufacturer's protocol. dsDNA was submitted to IIGB, UCR, where it was subjected to fragmentation, end-repair and PCR amplification using the Encore NGS Multiplex System 1 (NuGen), following the manufacturer's protocol. Further, samples were multiplexed in groups of 4 and were subjected to paired-end sequencing in 2 lanes on the HiSeq2000 (Illumina).

Illumina Data Processing.

Whole Flies. Data obtained from IIGB, UCR were de-multiplexed based on barcode sequences using custom written PERL scripts. Reads matching ribosomal RNA sequences (using the BLAT alignment program (Kent 2002)) were removed from further analysis, since they likely were degradation products that had contaminated the library. The remaining reads obtained from Illumina were aligned against the *Drosophila melanogaster* genome, Berkeley *Drosophila* Genome Project (BDGP) assembly release 5. (Trapnell et al. 2009), using default parameters. Expression levels of known *Drosophila melanogaster* transcripts were estimated using RPKM values (Mortazavi, Williams et al. 2008). Differential gene expression analysis was performed using the DEseq R-package (Anders et al. 2010) comparing control versus RNAi libraries. A p-value cut-off of < 0.1 was used, due to lack of replicates; p-values instead of p-adjusted were used for analysis. Only transcripts with reads ≥ 10 in at least one of the libraries were used for analysis (Illumina, 2011).

Fly Heads. Illumina data was analyzed as described for whole fly data.

Fly Corpora Allata. Data from the two lanes, technical replicates, were combined to increase the sequencing depth (Bonizzoni, Dunn et al. 2011). Further analysis was done as described for whole fly and head data, using the same cut-offs.

Data Analysis. Gene identifiers for differentially expressed genes were uploaded to www.flymine.org and chromosomal distribution, tissue distribution and gene names were

converted into database identifiers (eg. FBgn0028738). A database identifier list was used for gene ontology enrichment analysis. GO terms identified by Flymine were uploaded on Orgin 8.1 and pie charts were generated.

Illumina Data Validation. Illumina data validation was done by quantitative PCR (qPCR). A total of 4 genes were chosen for qPCR analysis. Body parts (25 fly heads, 10 CAs and 50 legs) were collected from each genotype separately by snap freezing flies in liquid nitrogen, except for CAs, which were extirpated under cold saline and transferred immediately into a 1.5 ml tube that was kept on dry ice. Total RNA was extracted from heads, CAs and legs using TRIZOL (Invitrogen) reagent using the manufacturer's protocol and was DNase treated using Turbo DNA-free (Ambion). Treated RNA was subjected to reverse transcription using the oligodT method with Superscript II (Invitrogen). qPCR was performed in triplicate for 2 biological replicates for head and one for CA and legs. Primers were optimized and standard curves were generated using a series of dilutions of cDNA to determine the efficiency of each primer pair. qPCR was run in a 25 µl reaction using SYBR Green reagent (Biorad), on a iQ5 system (IIGB, University of California, Riverside). Primers were tested for primer dimers using melting curves for each pair; only pairs with no primer dimers were used for analysis. The threshold was set manually within the linear amplification range and the cycle threshold value (Ct value) was determined. Based on Illumina data, *Actin5c* changed least amongst house keeping genes; therefore it was used as the reference gene for qPCR analysis. Fold change was determined using the Pfaffl equation (Pfaffl 2001).

***Doublesex* exon Cloning and Primer Design.** Primers were designed in a way to detect clear differences in product size with and without the exon. PCR product of size ~150 bp was expected without the exon and ~1 Kbp with the exon in between known male exons. RNA was extracted from 50 CS heads using TRIZOL reagent (Invitrogen) and DNase treatment (Qiagen) was done in order to remove any genomic DNA contamination. cDNA synthesis was done using the Oligo dt method and Superscript II (Invitrogen). PCR product was run on a 2% agarose gel and product size was determined by running the product with Gene Ruler ladder (Fermentas). Product was cloned using standard cloning methods. Extraction of the band of interest was done (Qiagen) and the product was cloned into a pJET vector using standard protocol. *E.coli* (DH5α-NEB) were transformed using the manufacturer's protocol, plated on agar (Fisher), and grown overnight to generate colonies. Individual colonies were checked for insertions using vector specific primers for colony PCR and colonies with positive insertions were grown overnight in 3 ml LB broth (Fisher). Minipreps were performed to extract plasmid from bacteria (5Prime) and DNA was sent for sequencing using vector specific forward and reverse primers to IIGB, UCR. Sequence quality was checked by determining the peak height on Chromas and BLAST2 was used to match the sequence with the genomic *dsx* sequence. The sequence matched 100% with the *dsx* intron. Splice junctions were checked manually to confirm the presence of a new *dsx* exon in males.

RESULTS AND DISCUSSION

Male flies use sensory cues, including visual, olfactory, gustatory and mechanosensory inputs for mate recognition during courtship behavior. Several genes involved in these sensory inputs are known to regulate courtship behavior in male flies (Greenspan and Ferveur 2000; Baker, Taylor et al. 2001; Billeter, Rideout et al. 2006; Villella, Hall et al. 2008). ETHR silencing leads to elevated levels of male-male courtship (Chapter 2) and altered patterns of gene expression. Male courtship regulatory genes are expected to be differentially expressed after ETHR-RNAi. This study focuses on those genes that are known to regulate male-courtship behavior and also other genes that might be enriched downstream of ETHR-RNAi that might be regulating male courtship behavior genes.

In order to elucidate molecular mechanisms underlying elevated male-male courtship, RNA-seq analysis of 3 samples (corpora allata, heads and whole flies) from adult males was done and differentially expressed genes were determined by comparing the ETHR-RNAi library to the control library. ETHRs were silenced using *Aug21-Gal-4,* which drives expression of dsETHR-RNA in the CA and a subset of central neurons. In order to describe altered gene expression patterns in the CA, this structure together with closely associated tissues (referred as CA sample/library in this study) was dissected out from adult males and libraries were prepared for gene expression profiles. In order to focus on gene expression changes in the nervous system and other head components, including the sensory system, a head sample was used for differential gene expression.

Whole body tissue was used for RNA-seq analysis to obtain an overall picture of genes from whole body and peripheral systems that might change as a result of ETHR silencing. Expression profiles of all three samples should cover most of the genes at all different locations and their tissue specific changes. To my knowledge, all gene expression profile studies performed thus far have focused on one tissue only. Genes are known to play stage and tissue specific functions (Boltz et al. 2007); they also are regulated in a tissue specific manner. During metamorphosis, tissue-specific gene expression and regulation takes place, where each tissue displays a unique response to ecdysone (Li et al. 2003). It is important to know how gene expression changes vary in a tissue-specific manner. This is the first study where gene expression changes are analyzed in 3 different samples.

Differentially expressed transcripts determined by DESeq and various cut-offs were used for data analysis. Plots were generated for all three samples, to visualize differential transcript expression; log2-fold changes were plotted against average number of reads mapped to the gene and adjusted for library size (Fig. 3-1). Each dot represents an individual transcript; transcripts that exceeded cut-offs are marked as red dots and those that did not are marked as black. Differentially expressed transcripts included 1419 (6.17%) from the CA library, 1650 (7.17%) from the head library and 1552 (6.74%) from the whole fly library. As a result of ETHR silencing, the highest number of genes observed to change are in male fly heads, which is the control center for behavioral coordination.

Transcripts that were not detected in either library were not plotted on the fold change scatter plots. Undetected transcripts numbered 4192 in CA libraries, 1266 in head libraries and 1443 in whole fly libraries. Differentially expressed genes from each sample were analyzed for transcription factors, genes involved in male courtship behavior, axon-guidance and genes interacting with doublesex-*dsx* and fruitless-*fru.* Lists of genes enriched as terms transcription factor, male courtship behavior and axon-guidance were generated from amigo.geneontology.com. Genes interacting with *dsx* and *fru* were determined by www.flymine.org. Genes related to male courtship behavior (60), 412 transcription factors (412), axon guidance related genes (195), *dsx* interacting (48) and *fru* interacting genes (10) were individually searched in differentially expressed genes from all three libraries.

Fly Male Corpora Allata.

Basic Sequencing Results. RNA-seq libraries were generated from 3-5 day old, individually raised adult *Drosophila melanogaster* males by dissecting flies and extracting corpora allata along with some closely associated tissue between ZT 7-9 and samples were processed and were divided to run into two lanes. Data from the two technical replicates were combined to increase sequencing depth (Bonizzoni et al. 2011). RNAi libraries were compared to control libraries to identify genes changing post ETHR silencing in whole flies. One replicate of the RNAi library generated 15,238,141 reads and the other replicate generated 9,203,833 reads. After combining the two replicates 9,164,753 reads mapped to the *Drosophila* genome (BDGP assembly release 5)..

14,561,826 reads were generated from one replicate of control library and the second replicate generated 9,136,725 reads, after combining the two libraries 9,104,911 reads mapped to the *Drosophila* genome (BDGP assembly release 5). This shows that both libraries had a similar ratio of ribosomal to non-ribosomal sequences.

This is the first report on the transcriptome of adult *Drosophila* male CA tissue. Apart from juvenile hormone synthesis genes, genes expressed in gut, and *Akh, from* corpora cardiaca, a few unannotated genes were highly expressed in CA tissue. *CG34220* has the highest number of reads (1251005) in the control CA library. It is a chitin peptidase precursor protein that is probably expressed in the gut. Unexpectedly, out of 174 male biased proteins thought to be expressed only in male accessory glands, 57 are expressed in male CAs (Table 3.1) (Ranz et al. 2003; EB Rodgers-Melnick et al., 2010). This is consistent with RNASeq results obtained from *Bombyx* male CA, where orthologs of male accessory gland genes also are expressed (Zitnan et al., in preparation). In the *Bombyx* study, the male CA transcriptome was obtained without contamination from surrounding tissue. Considering the homology in gene expression pattern in insects, it is therefore reasonable to conclude that male accessory gland genes are expressed in *Drosophila* male CAs. Juvenile hormone mutants express low levels of male accessory gland proteins in male accessory glands (Whalen et al. 1986; Shemshedini et al. 1990). These genes are predicted to play male reproductive organ-specific functions, such as spermatogenesis and reproduction. Presence of 57 male accessory gland-specific genes in *Drosophila* male CAs suggests a new functional role for this organ.

A few gustatory receptors are expressed in the CA control library. This may be due to presence of gut tissue in CA samples. It is interesting to see internal expression of Grs, whose presence may sense levels of sugar and toxins circulating in hemolymph. Grs that were detected (>10 reads) in the CA control library are: *Gr43a, Gr59a, Gr59b, Gr59c, Gr59d, Gr47a* and *Gr39a*. *Gr43a* is a fructose receptor (Sato et al. 2011). *Gr59a, Gr59b, Gr59c* and Gr59*d* occur in the taste receptor family, but have no known ligand or function as yet. Micro-RNAs, including *mir-2a-1, mir-2b-1, mir-14, mir-2a-2, mir-281b, mir-307* and *mir-308* were also detected in control CAs. Since the CA sample includes small amounts of surrounding tissue, many of these genes may come from corpora cardiaca or gut. However internal expression of male accessory gland specific genes in the CA opens a new avenue for studying functions of these genes.

Differential Gene Expression. DEseq analysis using a p-value cut-off of 0.1 and minimum number of reads in at least one library ≥ 10 yielded a total of 1419 transcripts corresponding to 997 genes changing in RNAi library as compared to the control library. 715 up-regulated transcripts correspond to 484 genes and 704 down-regulated transcripts correspond to 513 genes. The log2-fold change distribution in up-regulated transcripts ranged from 0.08 to 10.31, out of which 11 transcripts are 7-10 fold up-regulated, 11 are 5-7 fold, 498 transcripts range from 1-5 fold change, 27 from 0-1, and 130 transcripts are only present in RNAi library (Fig. 3-2). The log2-fold change distribution in down-regulated transcripts ranges from 0.03 to 9.95, where 14 transcripts are 7-10 fold down-regulated, 21 transcripts change between 5-7 fold, 464 transcripts between 1-5, 26

transcripts range from 0-1 fold down-regulated and 179 transcripts are present only in the control library. The large number of genes changing in response to ETHR RNAi in male CAs indicates a significant functional role for ETHRs in adult male flies.

Chromosomal Distribution. Genes changing as a consequence of ETHR-silencing were uploaded on www.flymine.org and the chromosomal distribution was determined for up-regulated and down-regulated genes. 484 up-regulated genes were uploaded, 483 were identified, whereas 1 gene remained unidentified. Amongst 483 identified genes, 96 mapped to chromosomal arm 2L, 132 mapped to 2R, 113 mapped to 3L, 76 mapped to 3R, 63 mapped to the X-chromosome and 1 mapped to chromosome 4. Out of 513 down-regulated genes, 506 were identified and 7 were not identified. Amongst 506 identified genes, 85 mapped to 2L chromosomal arm, 155 mapped to 2R, 73 mapped to 3L, 105 mapped to 3R, 3 mapped to the fourth chromosome and 60 mapped to X-chromosome; positions of 22 genes were not located (Fig. 3-3). Detailed analysis of gene locations shows that the down-regulated genes on chromosome 2R and 3L belong mostly to a specific location on the chromosome (Fig. 3-4). Clusturing of differentially expressed genes as a result of ETHR-RNAi suggests that ETHR-RNAi affects chromatin organization.

Tissue Distribution. According to Flymine analysis, genes changing as a consequence of ETHR silencing also are expressed in various tissues and body parts including heads. Amongst the 484 up-regulated genes, a large number of genes are expressed in heads (111), 128 in brain, 120 in hindgut, 105 in male accessory glands, 108 in midgut, 168 in

ovaries and 138 in testis (Fig. 3-5). Amongst the 954 down-regulated genes, most genes (141) are also expressed in head, 175 in brain, 140 in the male accessory glands, 135 in ovaries, 93 in testis and 69 and 79 in hindgut and midgut, respectively. It is important to note that ETHR silencing causes down-regulation of most genes previously thought to be exclusively expressed in male accessory glands. Since ETHR silencing causes elevated male-male courtship (Chapter II), it is suspected that down-regulation of genes highly expressed in the reproductive organs may contribute to changes in male courtship behavior.

Differentially Expressed Genes Based on GO Categories. Gene ontology analysis for both up-regulated and down-regulated genes, based on biological process, cellular function and molecular function, was done on www.flymine.org using the Holm-Bonferroni multiple hypothesis test correction at $p < 0.05$. Data was uploaded on www.flymine.org and GO terminologies were generated for each dataset. Genes falling into each category were transferred to an Excel file and pie charts were generated using Origin 8.1. Since ETHR silencing leads to courtship defects in *Drosophila* adult males, reproduction-related genes are expected in the enrichment analysis.

GO of Up-regulated Genes in the CA Library. None of the terms were enriched at $p < 0.05$. Since the primary focus of this study is to identify genes involved in male courtship behavior after ETHR silencing, a less stringent statistical test for multiple hypothesis at $p < 1.0$ was used to find genes associated with reproduction, male courtship behavior and genes known to be affected by general RNAi. Using the p-value,

reproduction, metabolic process, defense response and nervous system development terms were found to be enriched. Defense response genes are known to be affected by RNAi (Whitehead et al. 2011) due to induction of the Toll pathway by RNAi machinery. In order to cover all genes known to be involved in male courtship behavior, a gene list was created from amigo.geneontology.org that included genes under the term "male courtship behavior". These genes each were checked individually in the differentially expressed gene list. There were 3 up-regulated genes known to be involved in male courtship behavior: *Gustatory receptor 33a (Gr33a), white (w), and fragile X mental retardation* (*Fmr1)*. An increased level of white gene expression is known to result in elevated male-male courtship behavior (Zhang et al. 1995). *Gr33a* knockout mutants also are known to display increased male-male courtship (Moon et al. 2009). This is inconsistent with our behavior results from ETHR-RNAi adult flies, which show increased male-male courtship. *Fmr1* is a selective RNA-binding protein that regulates translation of target mRNAs in mammals (Brown et al. 2001). It is known to be involved in glutametergic synaptic transmission (Chang et al. 2008). *Fmr1* mutants display reduced courtship behavior towards virgin females and immature males; these flies are known to have elevated expression of *futsch* (Dockendorff et al. 2002; Chang et al. 2008). ETHR-RNAi elevates male-male courtship, and up-regulates *fmr1* and down-regulates *futsch* by 1.54 and 0.97 fold. As a result of ETHR-RNAi, *juvenile hormone acid methyltransferase* (*jhamt)* and *juvenile hormone-inducible protein* (*jhl-1*) are up-regulated by fold changes of 1.54 and 1.87, respectively (Huang et al. 2011). Up-

regulation of genes associated with JH synthesis suggests the possibility that JH levels are elevated in ETHR-silenced males.

GO of Down-regulated Genes in the CA Library. Enriched terms found in down-regulated genes are expected to be related to reproductive behavior. Biological process-based GO enrichment analysis yielded 43.37% (72) of genes involved in reproduction, 5.42% (9) involved in post-mating behavior, 3% (5) genes involved in polytene chromosome puffing and 3.61% (6) involved in sperm competition. Out of 174 known male biased genes, 55 expressed in male accessory glands are present in male CAs and are down-regulated after ETHR-RNAi (Fig. 3-6a). JH is known to play a role in male accessory gland protein accumulation; presence of 55 genes specific for male accessory glands in CA indicates a direct relation in regulation of these proteins (Whalen et al. 1986; Shemshedini et al. 1990). 5 genes are classified as polytene chromosome puffing genes; these include 4 heat shock protein genes (*Hsp70Ba, Hsp70Bbb, Hsp70Bb, Hsp70Bc*) and *poly-(ADP-ribose) polymerase (parp),* which are known to have histone binding properties. Since ETHR-RNAi elevates male-male courtship, genes involved in reproduction were expected to be enriched. As expected, a majority of genes (43.37%) are involved in reproduction. Genes termed as "male courtship behavior" on amigo.geneontology.com were observed to be down-regulated after ETHR-RNAi (*maleless (mle), paralytic (para)* and *slowpoke (slo)*). *mle* is a dosage compensation gene that acts as a RNA/DNA helicase; it is also known to have a ATPase activity (Lee et al. 1997). It is also known to affect exon splicing of the *para* sodium channel gene Reenan et

al. 2000). *slo* is a calcium-activated potassium channel, that is involved in male courtship behavior and specifically the male courtship song (Peixoto et al. 1998). Mutation of *slo* causes a reduction in transmitter release at the neuromuscular junction. There are 16 transcription factors down-regulated in CAs after ETHR-RNAi. These include, *acj6, Dad, dl, bi, br, CG9876, CG42741, fd59A, nub, otp, Pcl, ptx1, svp, TFAM, tsh* and *Usf.* Down-regulated genes *Dscam3*, multiplexin (*mp*), *acj6* and *Dad* are involved in axon guidance. Down-regulation of the axon guidance gene *acj6* is also known to affect synaptic targeting and odorant receptor gene expression (Certel et al. 2000; Ray et al. 2007). In immature stages of insects, *broad* (*br*) is induced by ecdysone (20E), and inhibited by the presence of juvenile hormone (JH). It is a key regulator in mediating crosstalk between the 20E and JH signaling pathways (Zhou et al. 1998; Zhou et al. 2002). Roles for *br* are not described yet, but roles for ecdysteroids and JH in adults have been described. Decreased ecdysteroid and JH levels results in elevated male-male courtship (Liu et al. 2008; Ganter et al. 2011). ETHR mediated *br* expression and male courtship behavior regulation suggests a role for *br* in male courtship behavior. Gustatory receptors *Gr59a, Gr59b, Gr59c* and *Gr59d* along with olfactory receptor *Or83a* and other odorant binding proteins- *Obp56f* and *Obp22a* are down-regulated, indicating effects of ETHR-RNAi on sensory systems. Other sensory genes like *Ac78C, stj, Rh2, sws, inaC, inaD, trpgamma* and *ana1* also are down-regulated. This suggests that neuronal circuitry associated with male-male courtship behvaior is affected as a result of ETHR-RNAi.

To identify genes related to male courtship, expression of *fru* and *dsx* and genes interacting directly with them were checked in the dataset. Both *fru* and *dsx* are not differentially expressed in the CA library. Screening for genes directly interacting with *fru* and *dsx* shows that *dorsal* (*dl*) interacting directly with *dsx* is down-regulated after ETHR-RNAi (Giot et al. 2003). Dorsal is a rel-family transcription factor involved embryonic development and the Toll signaling pathway (Valanne et al. 2011); unlike other immune response genes it is down-regulated in ETHR-RNAi male CAs.

Three GO terms were enriched under the GO category "cellular component": extracellular region, extracellular region part and extracellular space (Fig. 3-6b). This reflects presence of structural components and enzymes, which are likely involved during social behavior. The terms enriched under GO category "molecular function" include oxidoreductase activity (12.94%), carboxylic acid binding (11.76%), and an additional 8 genes in all remaining 8 terms (Fig. 3-6c). These data implicate involvement of ETHRs in coordination of metabolic pathways.

Overall, differentially expressed genes in the CA appear to be affected immediately downstream of ETHRs. Down-regulated genes enriched in reproduction and differential expression of male courtship genes indicates involvement of ETHRs in male courtship behavior. Interestingly, one gene involved in juvenile hormone synthesis is up-regulated after ETHR silencing: *jhamt*, the enzyme which esterifies JHacid. *jhI-1* also is up-regulated after ETHR-RNAi, while *br* is down-regulated. JH is known to promote *jhI-1*expression and represses expression of *br*, which is a transcription factor regulating

metamorphosis downstream of JH (Fig.3-19). Although it is suggested that increased JH esterase results in elevated male-male courtship (Liu et al. 2008), precise roles of JH in regulation of male courtship behavior remain to be elucidated.

Transcripts Found Only in the CA RNAi Library. 130 transcripts were found only in RNAi male CAs with read counts ranging from ~63 to 11, after normalization for library sizes. Functionally, these transcripts are involved in metabolism, RNA splicing and reproduction.

Transcripts Found Only in Control Library. 173 transcripts found in the control library were eliminated entirely after ETHR-RNAi. 27 of these are involved in reproduction. Most of these transcripts are male accessory gland-specific. a*cj6* transcripts, involved in axon guidance and courtship behavior, are also undetectable in CA tissue as a result of ETHR-RNAi. This suggests re-wiring of neuronal circuitry as a result of ETHR silencing.

Fly Heads.

Basic Sequencing Results. RNA-seq libraries were generated from 3-5 day old, individually raised adult *Drosophila melanogaster* males by flash freezing flies and quickly collecting heads between ZT 7-9. The RNAi library was compared to the control library to identify genes changing post-ETHR silencing in whole flies. The RNAi library generated 21,906,100 reads, out of which 68.75% (15,059,948) of the reads mapped to the *Drosophila* genome (BDGP assembly release 5). For the control library, 18,608,383

reads were generated, out of which 29.46% (5,482,217) reads mapped to the *Drosophila* genome (BDGP assembly release 5).

Differential Gene Expression. DEseq analysis using a p-value cut-off of 0.1 and minimum number of reads in at least one library ≥ 10 yielded a total of 1650 transcripts corresponding to 1157 genes changing in RNAi library as compared to control library. 263 transcripts are up-regulated, corresponding to 203 genes and 1387 transcripts are down-regulated, corresponding to 954 genes. The log2-fold change distribution in up-regulated transcripts ranges from 0.70 to 7.29 (Fig. 3-7), out of which 1 transcript is 7-10 fold up-regulated, 142 transcripts range from 1-5 fold change, 109 from 0-1, and 11 transcripts are only present in RNAi library. The log2-fold change distribution in down-regulated transcripts ranges from 0.63 to 8.31, where 10 transcripts are 7-10 fold down-regulated, 44 transcripts are between 5-7, 886 transcripts between 1-5, 315 transcripts range from 0-1 fold down-regulated and 132 transcripts are present only in the control library. In heads, unlike CA, more genes are down-regulated than up-regulated as a result of ETHR-RNAi. The high number of genes changing as a consequence of ETHR RNAi in male heads indicates significant functional roles for ETHRs in adult male flies.

Chromosomal Distribution. Genes changing downstream of ETHR-silencing were uploaded on www.flymine.org and chromosomal distribution was determined for up-regulated and down-regulated genes. Of the 203 up-regulated genes that were uploaded, 202 were identified. Amongst 202 identified genes, 39 mapped to chromosomal arm 2L, 59 mapped to 2R, 24 mapped to 3L, 28 mapped to 3R, 42 mapped to X-chromosome and

3 mapped to chromosome 4. Similarly, out of 954 down-regulated genes, 941 were identified and 13 were not identified. Amongst 941 identified genes, 261 mapped to 2L chromosomal arm, 226 mapped to 2R, 144 mapped to 3L, 214 mapped to 3R, 5 mapped to fourth chromosome and 88 mapped to the X-chromosome (Fig. 3-8).

Tissue Distribution. Genes changing as a consequence of ETHR silencing in fly heads belong to various different tissues. Amongst the 203 genes up-regulated, a high number of genes are from heads (117), 46 are also present in brain, 51 in hindgut, 26 also in male accessory glands, 50 in midgut, 32 in ovaries and 23 are also present in testis. (Fig.3-9). Amongst the 954 down-regulated genes in heads, 112 genes were down-regulated in head, 101 are also expressed in brain, 114 in male accessory glands, 177 in ovaries, 339 in testis and 221 and 197 are also expressed in hindgut and midgut, respectively. It is important to note that most down-regulated genes in heads are also known to be expressed in testis. Also, many hindgut (221) and midgut (197) genes were down-regulated as a result of ETHR silencing. It is possible that ETHRs play physiological roles other than regulation of male-male courtship in adult flies. Since physiological effects were not tested in ETHR silenced flies, it is unclear how down-regulation of hindgut and midgut genes affects ETHR related male courtship behavior phenotype.

Differentially Expressed Genes Based on GO Categories. Gene ontology analysis for both up-regulated and down-regulated genes in heads after ETHR-RNAi was done as described for CA data. RNAi is known to activate the Toll signaling pathway, and consequently defense response genes are expected to be enriched in up-regulated genes.

Since ETHR silencing results in elevated male-male courtship, down-regulated genes are expected to be enriched in the GO term "reproduction".

GO of Up-regulated Genes in Head Library. Similar to the CA library, terms enriched under the "biological process" GO category include defense response, anti-bacterial immune response and response to stress. Unlike CA library, polysaccharide catabolic process and aminoglycan catabolic process terms are enriched (Fig. 3-10). This suggests that ETHR-RNAi has an effect on catabolic genes in *Drosophila* male heads. Surprisingly, *ETHR-B* is up-regulated 2.01 log2-fold with a p-value 0.05. Since *Aug-21-Gal-4* is not specific for CA, we expected ETHRs to be down-regulated in the head sample. A p-value of 0.05 suggests that the possibility of this up-regulation is an artifact and a biological replicate will increase confidence about altered expression levels. It is also possible that we are detecting dsRNAs that are produced as a result of RNAi. In order to resolve this, qPCR was done using primer specific for non-dsRNA region on two biological replicates.

One term was enriched under the GO category "cellular component". Similar to CA, extracellular region is enriched, indicating presence of structural components and enzymes. Molecular function-based GO enriched one term, N-acetylmuramoyl-L-alanine amidase activity. This indicates up-regulation of genes involved in hydrolysis of the link between N-acetylmuramoyl and L-amino acid residues in bacterial cell-wall glycopeptides. Up-regualted bacterial cell wall hydrolysis related genes indicate increased anti-bacterial defense response.

GO of Down-regulated Genes in Head Library. Enriched terms found in head down-regulated genes were not similar to CA. The biological process category did not include reproduction as enriched terms. Terms that were enriched include cellular component organization, organelle organization, chromatin organization, sarcomere organization, acetyl-CoA metabolic process and electron transport chain. 94 genes down-regulated in heads after ETHR-RNAi in the CA are histone protein genes; these are the genes known to be involved in organelle organization (Fig. 3-11a). Clustering of down-regulated genes in the CA library indicated that ETHR-RNAi might affect chromatin organization, and enrichment of term chromatin organization strongly indicates that ETHRs could be involved in chromatin organization. On the other hand, this could be a general RNAi response. Acetyl-CoA is a precursor for juvenile hormone and down-regulation of genes involved in acetyl-CoA metabolism suggest that juvenile hormone production might be affected due to ETHR-RNAi (Bellás et al. 2005). Juvenile hormone is known to play a role in male-male courtship (Liu et al. 2008). Similar to CA data, down-regulated genes include many male specific genes, including accessory gland proteins and male specific proteins.

In the head down-regulated genes, cellular component enriched terms include intracellular organelle and macromolecule complex, indicating presence of structural components downstream of ETHR-RNAi (Fig. 3-11b). ETHRs in the immature stages, larval and pupal, of insect life are known to regulate ecdysis behavior. During this

behavior, ETHRs regulate neuromuscular activities. Involvement of ETHRs in muscles suggests its possible regulatory role, which is carried over from the immature stages.

Terms enriched under molecular function include ~50% of the genes as "DNA binding proteins". These include 94 histone protein genes, transcription factors and other genes such as *Pms2, pan, Cdc6, Taf13, HP6, dj, Thd1* and *E(bx)* (Fig. 3-11c). This suggests that ETHRs might be involved in chromosome organization and gene regulation. The CA data also show clustering of down-regulated genes at 2 different chromosome locations, indicating possible involvement of ETHRs in chromatin assembly. Enrichment of GO term "DNA binding" and differential expression of 94 histone protein genes suggests that ETHRs are involved in chromatin organization.

In order to identify genes involved in male courtship behavior, 60 genes associated with the term "male courtship behavior" on amigo.geneontology.com were searched individually. *dsx* is the only gene involved in male courtship behavior that is down-regulated in heads after ETHR-RNAi in CA. *dsx* mutation diminish male courtship behavior towards females. ETHR-RNAi increases male-male courtship behavior and down-regulates *dsx* in male head. Since a significant number of transcription factors changed in CAs, all 412 genes named as TFs on amigo.geneontology.com were searched individually; 11 TFs were found to be down-regulated and 5 were up-regulated. *Dad, dsx, trx, CG11617, CG42741, cic, CG9876, Lmpt, mud, Pdp and twi* are downregulated and *Lim1, CG30080, oc, TfIIIB and vri* are up-regulated. Daughters associated with *dpp (Dad*) is known to play a role in regulating *dpp,* overexpression of *Dad* blocks *dpp*

(Tsuneizumi et al. 1997). *dpp* is known to be involved in the JH synthesis pathway. *Dad, dsx* and *trx* also are involved in axon guidance, hence 195 genes involved in axon guidance were individually searched. Three genes, *beat-IIa, daw and jeb* are up-regulated, whereas 6 axon guidance genes (*CadN, Gyc76C, Mical, Sema-1a, tutl* and *unc-5*) were down-regulated. *Sema-1a* is a semaphorin gene involved in synapse formation in giant fiber neurons (Murphey et al. 2003). Genes from the semaophorin family are known to interact with *plex-A/B* genes, which also play a role in synapse formation.

Transcripts Found Only in the RNAi Library. A few transcripts were expressed in the RNAi library, but not detected in the control library. Read counts of these 11 transcripts range from ~157 to 43, after normalization for library sizes. Female sterile (1) young arrest *(fs(1)Ya-RA*) is a chromatin assembly gene which is highly expressed in adult female ovaries, specifically nurse cells and oocytes and promotes embryogenesis and oogenesis (Lin et al. 1989). *alphaPs5-RA* is an integrin precursor, predicted to be involved in cell adhesion. Functions of the remaining 9 transcripts are unknown.

Transcripts Found Only in the Control Library. About 132 transcripts were expressed in control libraries, but not after ETHR-RNAi. Read count of these transcripts range from ~194 to 11, after normalization for library sizes. These include 23 histone protein transcripts, 3 gustatory receptor transcripts (*Gr59b, Gr59c* and *Gr59d),* and 2 yippee interacting protein-3 (*yip-3*) transcripts. *yip-3* is an adult male specific gene which is highly expressed in testis; it is involved in proteolysis with threonine-type endopeptidase activity. Down-regulation of this gene after ETHR-RNAi in adult heads suggests a

functional role in male courtship behavior. 4 transcripts (*msopa-RA, Acp98AB-RA, BG642163-RA* and *lectin-29Ca-RA)* are accessory gland related proteins involved in reproduction, of which *msopa-RA, BG642163-RA* and *lectin-29Ca-RA* are thought to be male accessory gland specific genes. *polo-RA and polo-RB* transcripts derived from the gene *polo* having protein serine/threonine kinase activity, are known to be expressed in ovary and testis. Six transcripts, *ms(2)35Ci-RA*-with unknown function*, klhl10-RA*-involved in oxidoreductase activity, *ACXA-RA* with adenylate cyclase activity and Casein kinase II β2 subunit (*CKIIbeta2-RA*) with protein kinase activity and *Tsp66A-RD*, *Tektin-A-RA,* Transition protein-like 94D *(Tpl94D-RA)*, solwind (*sowi-RA*), *ACXA-RA* and *CKIIbeta2-RA* are male specific transcripts, found specifically in adult testis. 2 transcripts are from tRNAs (*tRNA:CR30201-RA* and *tRNA:CR30202-RA)* and 2 transcripts Niemann-Pick type C-2e (*Npc2e-RB)* and *Lvpl-RA* are midgut specific transcripts. TurandotZ *(TotZ-RA)* is known to be expressed mostly in pupal stage, but the function is unknown. The remaining 85 transcripts are unannotated.

Whole Flies.

Basic Sequencing Results. RNA-seq libraries were generated from 3-5 day old, individually raised adult *Drosophila melanogaster* males by flash freezing whole flies between ZT 7-9. The RNAi library was compared to the control library to identify gene changes in expression patterns following ETHR silencing in whole flies. The RNAi library generated 7,651,370 reads, out of which 86.56% (6,622,833) reads mapped to the *Drosophila* genome (BDGP assembly release 5) and 8,161,141 reads were generated

from control library, out of which 87.93% (7,175,187) reads mapped to the *Drosophila* genome (BDGP assembly release 5). Since the libraries were multiplexed, a similar number of reads mapping from two libraries shows that there was no adapter bias involved. Around 87% reads mapped from both the libraries, showing that both libraries had a similar ratio of ribosomal to non-ribosomal sequences.

Differential Gene Expression. DEseq analysis using a p-value cut-off of 0.1 and a minimum number of reads in at least one library ≥ 10 yielded a total of 1537 transcripts, corresponding to 1155 genes changing in the RNAi library as compared to the control library. 929 up-regulated transcripts correspond to 729 genes and 608 down-regulated transcripts correspond to 426 genes. The log2-fold change distribution in up-regulated transcripts range from 0.56 to 6.94 (Fig. 3-12), of which 17 transcripts are 5-7 fold, 339 range from 1 to 5 fold, 556 from 0-1 fold up-regulated, and 17 transcripts are only present in RNAi library. The log2-fold change distribution in down-regulated transcripts ranges from 0.56 to 6.92, where 12 transcripts are 5-7 fold up-regulated, 170 transcripts between 1-5 fold, 415 transcripts between 0-1 fold down-regulated, and 26 transcripts are present only in the control library. The high number of genes changing in response to ETHR RNAi in males indicates a likely functional role of ETHRs in adult male flies.

Chromosomal Distribution. Genes changing in response to ETHR-silencing were uploaded on www.flymine.org and chromosomal distribution was determined for up-regulated and down-regulated genes. Of the 729 up-regulated genes that were uploaded, 724 were identified and 5 genes were unidentified. Amongst 724 identified genes, 123

mapped to chromosomal arm 2L, 179 mapped to 2R, 121 mapped to 3L, 167 mapped to 3R, 109 mapped to the X-chromosome while the location of one of the genes remained unidentified. Out of 426 down-regulated genes, 423 were identified and 3 were not identified. Amongst 426 identified genes, 83 mapped to 2L chromosomal arm, 80 mapped to 2R, 97 mapped to 3L, 88 mapped to 3R, 19 mapped to chromosome 4, while 47 mapped to the X-chromosome (Fig. 3-13).

Tissue Distribution. Genes changing as a result of ETHR silencing in whole flies belong to a variety of different tissues. Amongst the 729 genes that were up-regulated, a high number of genes are also expressed in midgut (259), hindgut (246) and head (225) (Fig. 3-14). 135 genes are also expressed in brain, 177 in the male accessory glands, 164 in ovaries and 79 in testis. Surprisingly, many differentially expressed genes are known to be expressed in ovaries (164) and fewer in testis (79). Amongst the down-regulated genes, most (144) are also known to be expressed in testis, 113 in brain, 100 in ovaries, 95 in head, 77 in hindgut, 71 in male accessory glands and 68 in midgut. It is important to note that ETHR silencing causes down-regulation of most genes in the testis. Since ETHR silencing causes elevated male-male courtship (Ref. Chapter II), it is possible that down-regulated genes in reproductive organs may be involved in male courtship behavior.

Differentially Expressed Genes Based on GO Enrichment Analysis. It is important to identify functional categories that are enriched in different tissues after ETHR silencing. Gene ontology analysis for both up-regulated and down-regulated genes, based on

biological process, cellular function and molecular function was done on www.flymine.org, using the Holm-Bonferroni multiple hypothesis test correction at $p < 0.05$. Data were uploaded on www.flymine.org and GO terminologies were generated for each data set. Genes identified in each category were transferred to an Excel file for creation of pie charts using Origin 8.1. Since ETHR silencing leads to male-male courtship, reproduction-related genes are expected in the enrichment analysis, as are immune-response genes as a general response to RNAi (Whitehead, Dahlman et al. 2011).

GO of Up-regulated Genes in the Whole Fly Library. In whole flies GO enrichment analysis as biological process terms show that 15.56% of up-regulated genes are involved in response to stress, 7.88% of genes are amine metabolic process genes, 7.68% of genes are multi-organism process genes and 7.27% of genes are involved in the defense response (Fig. 3-15a). Other up-regulated genes include those involved in the immune response, including bacterial and microbial response genes. Interestingly, 6 genes corresponding to 1.21% of all up-regulated genes are involved in chromosomal puffing.

ETHR silencing involved use of a symUAS-ETHR construct, which makes a dsRNA against ETHRs. As expected, ETHR-RNAi leads to a high number of up-regulated immune response genes. dsRNA constructs that contain more than 30 base pairs act as potent activators of the innate immune response (Whitehead, Dahlman et al. 2011). Most up-regulated genes (15.56%, 77 genes) are those involved in the stress response. Six up-regulated genes were classified as chromosome puffing genes, known to

play roles in transcriptional regulation. As a result of ETHR silencing, 11 heat-shock protein genes were up-regulated, out of which 6 are associated with chromosome puffing, including *Hsp70Aa*, *Hsp70Ab*, *Hsp70Ba*, *Hsp70Bb*, *Hsp70Bbb* and *Hsp70Bc*.

According to GO category "cellular component" enrichment analysis, 143 genes (28.77%) are enriched under the GO term cytoplasmic part, 13.08% as extracellular region and 11.47% as mitochondrion (Fig. 3-15b). It is interesting that cytoplasmic part and extracellular region are enriched in this dataset, indicating involvement of enzymes and structural components in courtship behavioral changes resulting from ETHR-RNAi. GO terms that are enriched under the "molecular function" GO category include oxidoreductase activity (40.43%) and structural constituents of ribosome (19.86%) (Fig. 3-15c).

Since the primary focus of this study is to identify molecular mechanisms underlying ETHR-silencing induced male-male courtship, a less stringent Benjamin and Hochberg test at $p < 0.1$ was used to analyze the data. After using this less stringent test, 34 genes involved in reproduction were identified in the up-regulated gene list (Table 3-2). Out of 34 genes listed as reproduction related genes, 2 are known to play roles in male courtship behavior. These include the *white* gene and the *doublesex* gene. Increased dosage of the *white* gene leads to elevated male-male courtship (Zhang et al. 1995; Hing et al. 1996). Doublesex is a transcription factor that plays an important role in *Drosophila* sex determination and is also known to regulate male courtship behavior (Rideout et al. 2010). Mutation of *dsx* results in diminished male courtship behavior towards females

which is a consequence of low detection of female pheromone by *Gr68a* (Bray et al. 2003). Up-regulation of *dsx* in the whole fly sample suggests extra sensitivity towards female pheromones; this pheromone may also be present in males at low levels. Furthermore, *Gr68a* is up-regulated in the CA library, suggesting extra sensitivity towards female pheromones, which could also cause elevated male-male courtship.

GO of Down-regulated Genes in the Whole Fly Library. After using the Holm-Bonferroni test for multiple hypothesis testing at $p < 0.05$, none of the terms were found to be enriched in down-regulated genes.

Upon using a less stringent Benjamin and Hochberg test at $p < 0.1$, 30 reproductive process genes were identified (Table 3-2). Of these 30 genes, 4 (*cac, dlg1, para* and *sphinx2)* are known to be involved in male courtship behavior. The gene *cac* is a voltage gated calcium channel involved in locomotory behavior, sensory perception of light and male courtship song. This gene interacts directly with *fru* (Florian 1976; Smith et al. 1998). Although no change in male courtship behavior towards females was observed, courtship song could be affected by ETHR-RNAi. This possibility remains to be tested. *Discslarge* (*dlg1*) is an epidermal growth factor present in all neurons. It plays roles in synaptic transmission and is known to be involved in male-male interactions (Ellis et al. 2011). *para* is a voltage-gated sodium channel known to play a role in courtship song. Similar to results obtained in CA library, *para* is down-regulated in heads. The *FOXP* gene, a *Drosophila* analog of human *FOXO* gene, known to be involved in acoustics and voice production, is down-regulated in whole flies. This

indicates that courtship song may be affected in ETHR-RNAi males. Experiments designed to measure courtship song parameters in ETHR-RNAi males are required to understand the role of ETHRs in regulating courtship song related genes. *Sphinx2* is a male specific serine-type endopeptidase activity gene expressed at high levels in the male accessory glands; it is predicted to be involved in male courtship behavior (Chen et al. 2011). Unlike other defense response genes, *sphinx2* is down-regulated in ETHR-RNAi males. 22 small nucleolar RNAs, which are non-coding genes associated with pseudouridation of RNA (Huang et al. 2004) were down-regulated, unlike 11 small nucleolar RNAs, which were up-regulated after ETHR-RNAi.

Transcripts Found Only in the RNAi Library. Seventeen transcripts were detected in the RNAi library but not in the control library. Four transcripts, *unpaired (upd3-RB), attacin (AttD-RA)* and *Cecropin* (*CecC-RA* and *CecB-RA*), are innate immune response genes. 3 transcripts *snoRNA:Psi28S-2292d-RA, snoRNA:Me28S-A2564-RA* and *snoRNA:Psi28S-2648-RA* are small nucleolar RNA. 4 are mitochondrial RNAs, out of which three, *mt:tRNA:S:AGY-RA, mt:tRNA:A-RA* and *mt:tRNA:V-RA* are tRNAs, while *mt:srRNA-RA* is small ribosomal RNA. One microRNA, *mir-308-RA* was also expressed and the remaining transcripts were unannotated.

Transcripts Found Only in the Control Library. Twenty six transcripts were detected in the control library, but not in the ETH-RNAi library. Two of the transcripts, *tRNA:Y1:28C-RA and tRNA:CR32761:Psi-RA,* are tRNAs. Eight transcripts are small nucleolar RNA. *Fad2-RA* is also present in this category; it is highly expressed in females

and known to play a role in pheromone production and courtship in females (Chertemps et al. 2006). *Allatostatin-cc* (*Ast-CC)* is also present in control libraries only; it is known to be expressed in the midgut. *Salivary gland secretion 1* (*Sgs1)* is also in this group and is an ecdysone-dependent gene present in salivary gland. It is expressed in very low levels in adults. Functions of the remaining genes are not known.

Comparison of Differentially Expressed Genes in CA, Head and Whole Fly. A total of 2901 genes changed after ETHR-RNAi, out of which 779 are specific for the CA library, 869 for the head library and 886 for the whole fly library. 104 are common to the CA and head libraries, 153 are common to the head and whole fly libraries and 79 are common to the whole fly and CA libraries. A total of 273 genes out of all the differentially expressed genes in at least two libraries change similarly in both the libraries, whereas changes in 95 genes are not consistent between the two libraries. 31 genes are changing in all three samples, 19 of which change similarly in all 3 tissues (Fig. 3-16). Amongst 2901 differentially expressed genes after ETHR-RNAi, 1270 are annotated and 1631 are unannotated. Of the 1270 annotated genes, 95 encode histone proteins, 68 encode TFs, 9 encode GRs, 10 encode Obps, 4 encode ORs, 6 encode Irs, 13 encode heat-shock proteins, 11 encode male specific Mst and 15 encode Acp genes, 21 encode Cytochrome p-450, 17 encode lethal genes with ATP-dependent DNA/RNA helicase activity, 12 encode Jonah genes, 30 encode mitochondrial genes, 9 encode micro-RNAs and 6 encode lectin genes.

Comparison of 2901 genes across the three samples shows tissue specific differential expression of genes. Interestingly, three transcripts of one gene, *ankyrin2* (*Ank2*) are differentially expressed in whole flies. *Ank2-RE* is up-regulated, and *Ank2-RI* and *Ank2-RO* are down-regulated, whereas all other transcripts are not differentially expressed. This indicates differences in regulation and function of each *Ank2* transcript. Ankyrin 2 is required for synaptic stabilization (Koch et al. 2008).

Gene ontology analysis on all differentially expressed genes was performed. Terms enriched under the GO category "biological process" included chromosome organization, chromatin assembly, chromatin organization, electron transport chain, and defense response (Fig.3-17a). As seen from individual tissue GO analysis, the majority of differentially expressed defense response genes are up-regulated, while chromatin organization genes are down-regulated. Terms enriched as "cellular components" include most genes from the mitochondrial respiratory chain complex-1. Down-regulation of these genes is known to increase lifespan in flies (Copeland et al. 2009), and levels of these genes decline with age (Ferguson et al. 2005). The term "intracellular non-membrane bounded organelle", which includes ribosomes, cytoskeleton and chromosome, were also enriched. 185 are from the extracellular region, which includes hormones and structural components, chromatin, contractile fiber part, myofibril and sarcomere terms were also enriched (Fig. 3-17b). Terms enriched as "molecular function" include NADH dehydrogenase and oxidoreductase genes (Fig. 3-17c). These genes are involved in metabolism and aging.

Quantitative PCR Analysis. A few genes were selected for qPCR analysis. *ETHR-A* (specific region), *ETHR-B* (specific region), *dsx, fru* and *Gr68a* were tested for expression in ETHR-RNAi flies. ETHRs are alternatively spliced GPCRs. Alternative splicing of the 4th exon results in expression of ETHR-A and ETHR-B. The ETHR-RNAi construct used in this study includes the first 3 exons, resulting in silencing of both receptors. ETHR-A and ETHR-B specific primers were used to determine the fold change in CAs and heads. Both ETHR splice variants were down-regulated, indicating the efficiency of RNAi. In CAs, ETHRs are down-regulated by ~2 fold, whereas in heads ETHRs were up-regulated. It was hypothesized that this might be due to the presence of dsRNAs that are generated as a result of RNAi. In order to resolve this, ETHR splice-variant specific primers were used. ETHR-B was determined to be down-regulated by 1.74 fold and ETHR-A was determined to be down-regulated by 3.15 fold. *dsx* was seen to be down-regulated after ETHR-RNAi, hence it was chosen for qPCR analysis. *dsx* is down-regulated by 2.865 fold in heads, which is consistent with RNAseq head data. Although *fru* does not change significantly in the RNAseq data, its fold change in *fru* was checked by qPCR, as it is known to play a role in male-male courtship behavior. *fru* is down-regulated by 1.11 fold in heads. *Gr68a* is up-regulated in CAs after ETHR-RNAi and hence it was chosen for qPCR analysis, since it is known to be expressed in legs, where it is predicted to detect female pheromone. qPCR was done on both CAs and legs. *Gr68a* is ~2 fold up-regulated in CAs and ~6 fold in legs (Fig. 3-18).

Candidate Genes Downstream of ETHRs Leading to Courtship Behavioral Change. Recently, microarray studies have been done on *Drosophila* male heads after male-male interaction and a list of socially-responsive genes was generated (Ellis et al. 2011). These authors showed that a total of 505 genes changed after male-male interaction, 240 of which were specific for male-male interaction as opposed to male-female interaction. In a search for genes involved in elevated male-male courtship resulting from ETHR-RNAi, all 240 of these genes were individually searched in our list of differentially expressed genes from all three samples.

This analysis showed that a total of 18 candidate genes changed in CA as a consequence of ETHR-RNAi, 15 changed in heads and 8 changed in whole flies, out of which 2 genes are common in heads and whole flies (Table 3-3). These genes, along with others summarized from each sample are suspected to be involved in ETHR-RNAi-related elevation in male-male courtship. Amongst the known male courtship behavior regulating genes that are differentially expressed after ETHR-RNAi, the sex-determination genes- *dsx and fru*, courtship song regulating genes- *cac, para, slo, mle* and *FoxP* along with a few other genes including *sphinx2*, *w* and *dlg1* (Table 3-4). Small changes in individual genes such as courtship song production genes and sensory system genes might not affect male courtship behavior. However changes in a combination of these genes together might elevate the phenotype. Some of the genes may be affected indirectly by ETHR-RNAi. GO categories enriched amongst differentially expressed genes after ETHR-RNAi suggests that ETHRs are involved in chromatin assembly,

probably due to changes in expression of histone genes or TFs. Based on these results, it is hypothesized that ETHRs alter gene expression by modifying chromatin organization, which in turn causes changes in expression of behavior-regulating genes leading to elevation of male-male courtship.

ETHR-RNAi results in differential expression of juvenile hormone-related genes. These changes are consistent with elevation in JH levels. A recent study indicates that overexpression of JH esterase (JHE), an enzyme involved in JH degradation, results in elevated male-male courtship (Liu et al. 2008). Unfortunately, quantification of male-male courtship was missing in this study, making it is difficult to compare the increase in courtship index to decreased JH levels. JH esterase converts JHIII to JH acid. JH acid is suspected to be functional signaling molecule in male adult (Bhaskaran, G., 1980). If this is true, overexpression of JHE could actually produce an increase in JH signaling (Fig. 3-19). It is therefore possible that ETHR-RNAi augments JH signaling to cause increased in male-male courtship.

Although a precise mechanistic explanation for how ETHRs lead to male-male courtship is not yet available, this study provides some insight into expression patterns of genes that are affected by ETHR-RNAi. After investigating all differentially expressed genes in ETHR-RNAi flies, it is hypothesized that ETHRs are allatostatic in function. Disruption of ETH signaling through ETHR-RNAi would therefore cause increased JH production. Differential expression of JH related genes and chromatin organization genes as a result of ETHR-RNAi led me to propose a model explaining regulation of male

courtship behavior by ETHRs (Fig 3-20). ETHRs regulate JH levels and chromatin organization, which affect sensory system genes and male courtship behavior. It is also possible that increased levels of JH cause chromatin reorganization or alternatively chromatin organization might be affecting JH levels. Overall, this model could explain the male-male courtship phenotype observed after ETHR-RNAi.

RNAseq Analysis Identifies a New *doublesex* Exon in Males. RNAseq analysis provides the opportunity of finding previously unidentified exons. RNAseq sequences were uploaded on www.modENCODE.org and reads mapping to the *dsx* were determined. *dsx* is a transcription factor which plays a role in sex determination. *dsx* is spliced in a sex specific manner, and transcripts in males and females differ in their last exon (Nagoshi and Baker 1990). These exons are specifically expressed in each sex and splicing is regulated by the TF *transformer. tra* is a transcription factor expressed in females that regulates sex-specific splicing (Ryner and Baker 1991). Interestingly, RNAseq data from male heads show reads mapping to the female specific exon (Fig. 3-21), consistent with a previous RNAseq study (Graveley, Brooks et al. 2011). When the number of reads is quantified, a relatively equal number of reads were found from male and female exons (Table 3-5). In a search for the female specific exon in males, primers were generated from ends of the 2 adjacent exons and PCR was performed in an attempt to amplify the female specific exon. Primers were designed in a way to see a clear difference in the product size with and without the exon. A PCR product of size ~150 bp was expected without the exon and ~1K bp with the exon in between known male exons.

A product of ~150 bp was seen on the gel as expected and an unexpected ~750 bp band also was seen on the gel. This indicated the presence of a novel exon for *dsx* in males. The gel band of 750 bp was cloned using standard cloning methods. Clones were grown and sequence quality was checked by determining the peak height on Chromas and BLAST2 to match the sequence with the genomic *dsx* sequence. The sequence matched 100% with the *dsx* intron. Splice junctions were checked manually to confirm the presence of a new *dsx* exon in males (Fig. 3-22).

SUMMARY AND CONCLUSION

In this study, differential expression of genes resulting from ETHR-RNAi was tested in three different samples. RNAseq analysis was done on each of the samples: corpora allata, head, and whole fly. This study is the first to report a corpora allata transcriptome. Juvenile hormone produced by corpora allata is known to regulate male specific proteins in male accessory glands. Transcriptome analysis of corpora allata revealed the presence of so-called male accessory gland-specific genes, suggesting a new possible role for male accessory gland proteins.

A total of 2901 genes showed altered expression, as a result of ETHR-RNAi. Of these, 779 are specific for the CA library, 869 for the head library and 886 for the whole fly library. 104 are common to the CA and head libraries, 153 are common to the head and whole fly libraries and 79 are common to the whole fly and CA libraries (Fig. 3-23). Out of all differentially expressed genes, a total of 273 genes change similarly in any two libraries, whereas 95 genes show dissimilar changes between the two libraries. 31 genes

change in all three samples, 19 of which change similarly in all 3 samples. Comparison of 2901 genes across three tissues shows a high degree of tissue-specific differential expression. Interestingly, three transcripts of *Ank2* are differentially expressed in whole flies. *Ank2-RE* is up-regulated, and *Ank2-RI* and *Ank2-RO* are down-regulated, whereas other *Ank2* transcripts are not differentially expressed. This indicates each *Ank2* transcript has a different functional role. *Ankyrin 2* is known to be required for synaptic stabilization (Koch et al. 2008) (Fig. 3-24).

Gene ontology analysis shows up-regulation of in immune response and metabolic pathway genes, and down-regulation of reproduction, courtship and chromatin organization genes. 2901 genes differentially expressed after ETHR-RNAi include a total of 95 histone protein genes, 68 transcription factors, 9 gustatory receptors, 10 odorant binding protein genes, 4 olfactory receptors, 6 Irs, 13 heat-shock protein genes, male specific 11 Mst and 15 Acp genes with serine type endo-peptidase activity, 21 Cytochrome p-450, 17 lethal genes with ATP-dependent DNA/RNA helicase, 12 Jonah genes, 30 mitochondrial genes, 9 micro-RNAs, 6 lectin genes and 1631 unannotated genes.

Overall, this study provides preliminary insights into mechanism underlying male-male courtship resulting from ETHR-RNAi. Differentially expressed gene analysis shows possible roles for ETHRs in chromatin organization and JH biosynthesis regulation, which potentially affects the sensory system. ETHR-RNAi alters chromatin organization and increases JH levels, which affects sensory system and increase male-

male courtship. The effect of JH in adult females has been extensively investigated, but little is known about functions of JH in males. A model is proposed that can be tested by investigating roles of JH in male courtship behavior and change in levels of JH after ETHR-RNAi. These experiments would help us understand the mechanism better.

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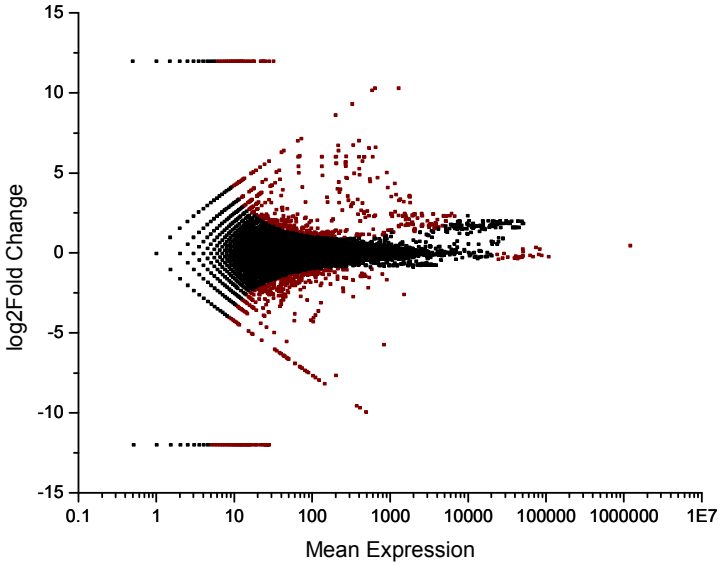
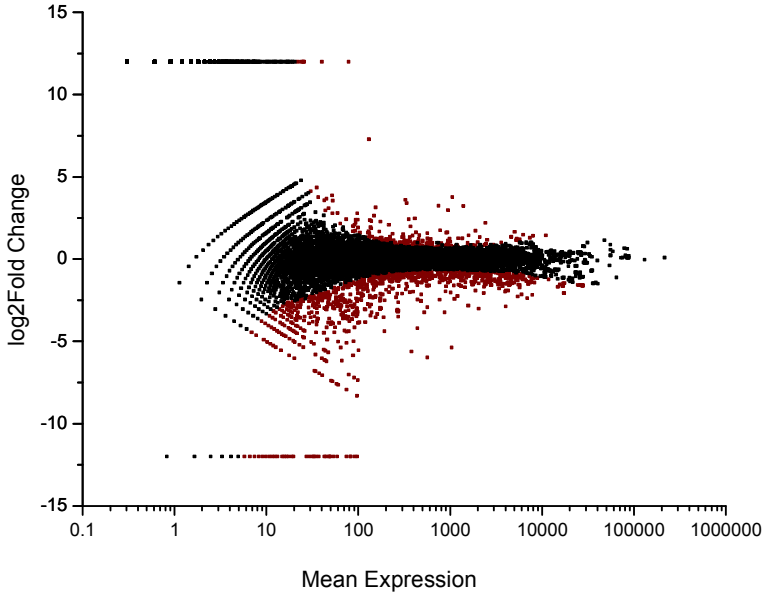
Fig 3-1. Tissue specific differential expression of transcripts relative to control library. Log2-fold change for each transcript plotted against mean expression of gene averaged across two libraries. All transcripts with >10 reads in at least one library and p-value < 0.1 represented as red, transcripts not detected in either libraries are not plotted and all other transcripts are plotted as black. a) Transcripts from corpora allata library showing 1419 differentially expressed transcripts. b) Transcripts from head library showing 1650 differentially expressed transcripts. c) Transcripts from whole fly library showing 1552 differentially expressed transcripts.

a)


15
10
5
0
-5
-10
-15
log2Fold Change
0.1
1
10
100
1000
10000
100000
1000000
1E7
Mean Expression

b)


15
10
5
0
-5
-10
-15
log2Fold Change
0.1
1
10
100
1000
10000
100000
1000000
Mean Expression

c)

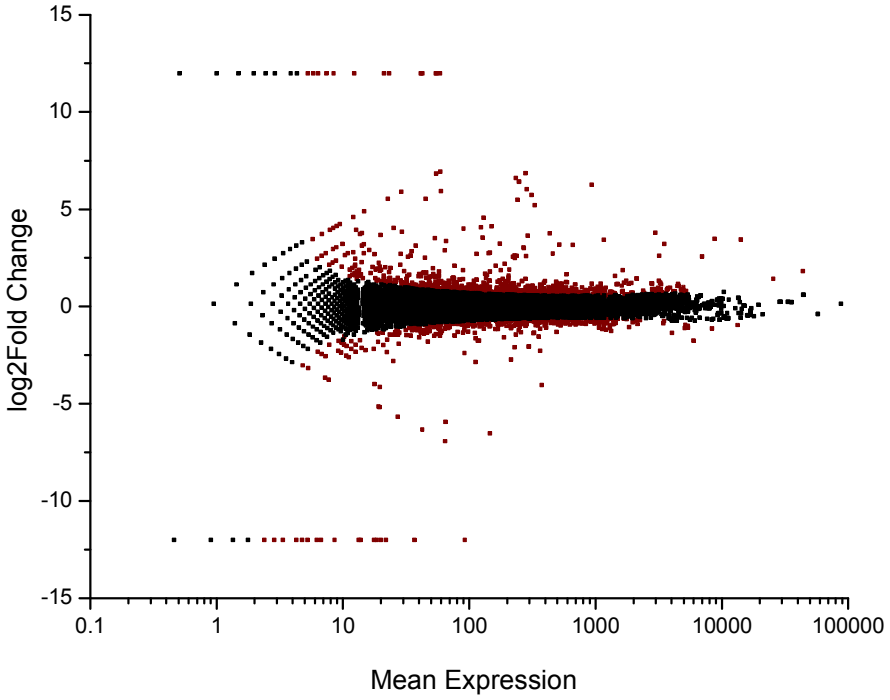

15
10
5
0
-5
-10
-15
log2Fold Change
0.1
1
10
100
1000
10000
100000
Mean Expression

Figure 3-1.

Figure 3-2. Fold change range of differentially expressed genes from corpora allata tissue.

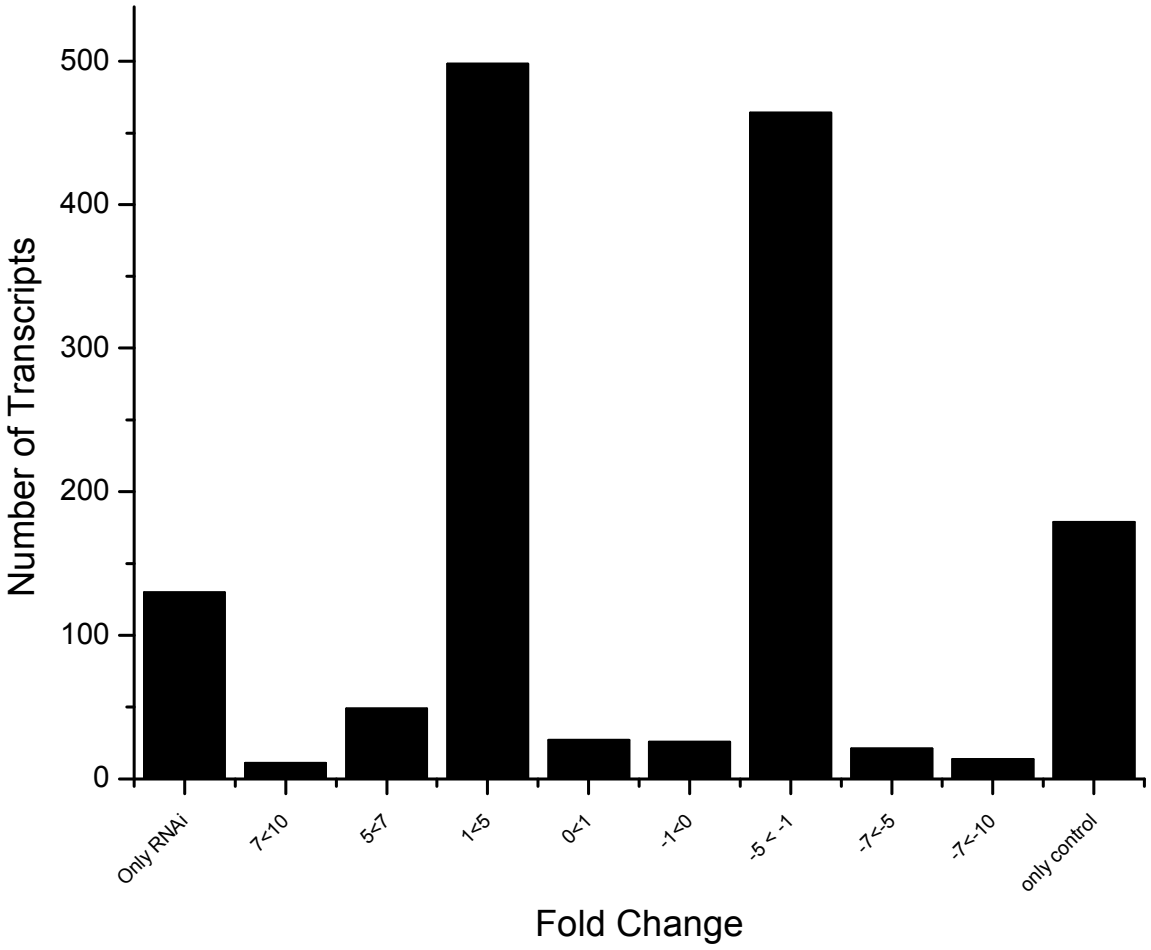

Number of Transcripts
500
400
300
200
100
0
Only RNAi
7<10
5<7
1<5
0<1
-1<0
-5 < -1
-7<-5
-7<-10
only control
Fold Change

Figure 3-2.

Figure 3-3. Chromosomal locations of differentially expressed genes from corpora allata tissue. L indicates left arm, R indicates right arm, numbers indicate chromosomal number and U indicates unidentified. Up-regulated genes are represented in black and down-regulated in red.

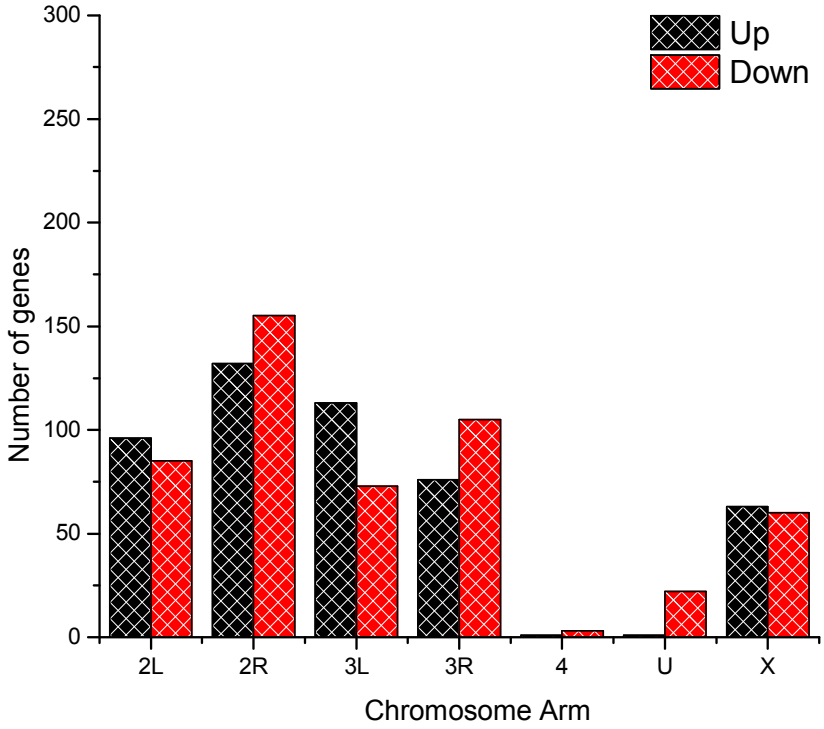
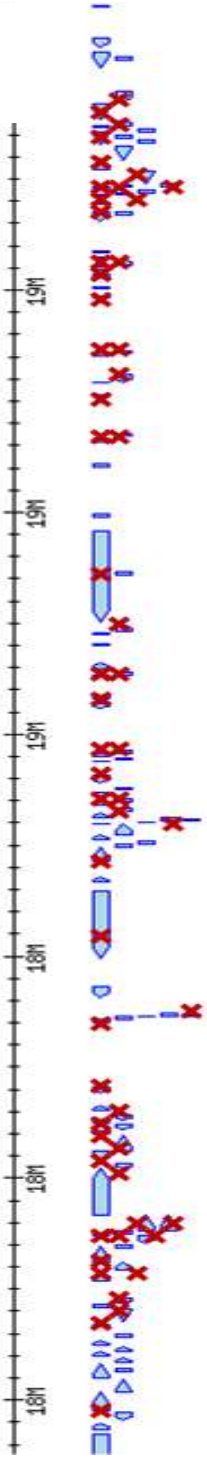

Up
Down
Number of genes
300
250
200
150
100
50
0
2L
2R
3L
3R
4
U
X
Chromosome Arm

Figure 3-3.

Figure 3-4. Clustering of differentially expressed genes from corpora allata.

a) Down-regulated genes from 2R chromosome arm between region18695809-19289832. b) Up-regulated genes from 3L chromosome arm between region11624495 -11968910.

a)



b)

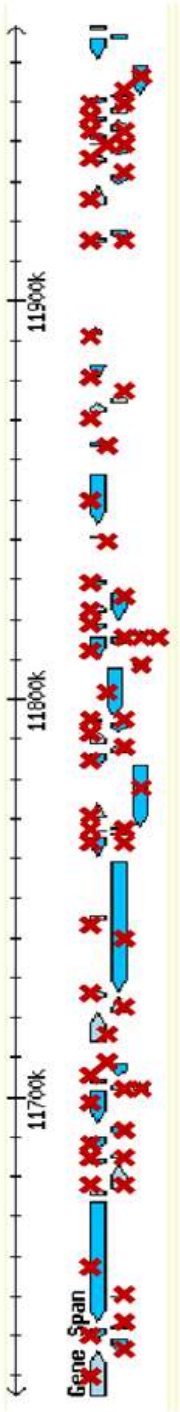


Figure 3-4.

Figure 3-5. Tissue distribution of differentially expressed genes from corpora allata tissue. Number of genes from each tissue represented as a bar graph. Up-regulated genes represented in green and down-regulated in yellow. MAG: male accessory glands.

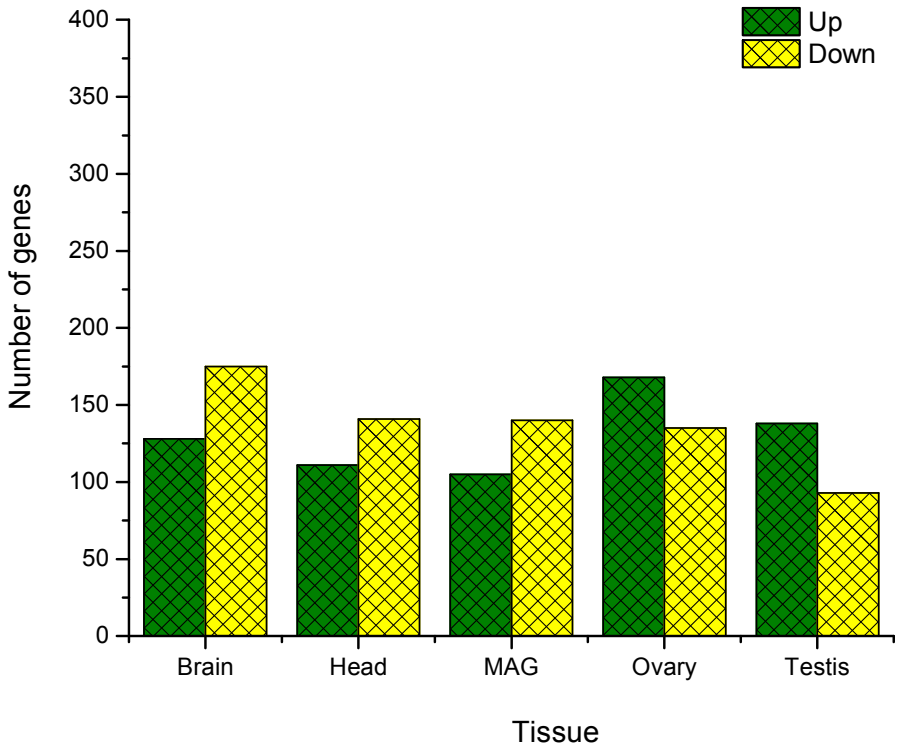
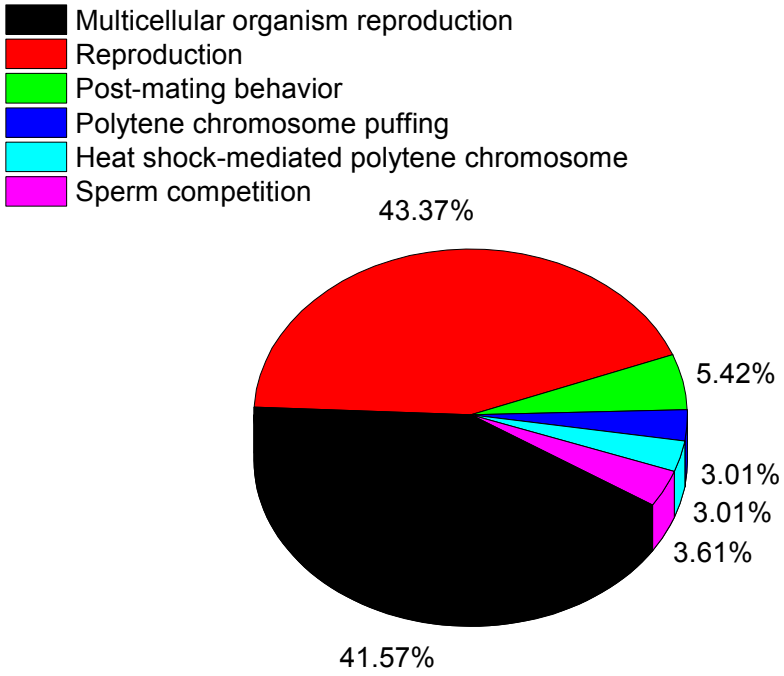

Up
Down
Number of genes
400
350
300
250
200
150
100
50
0
Brain
Head
MAG
Ovary
Testis
Tissue

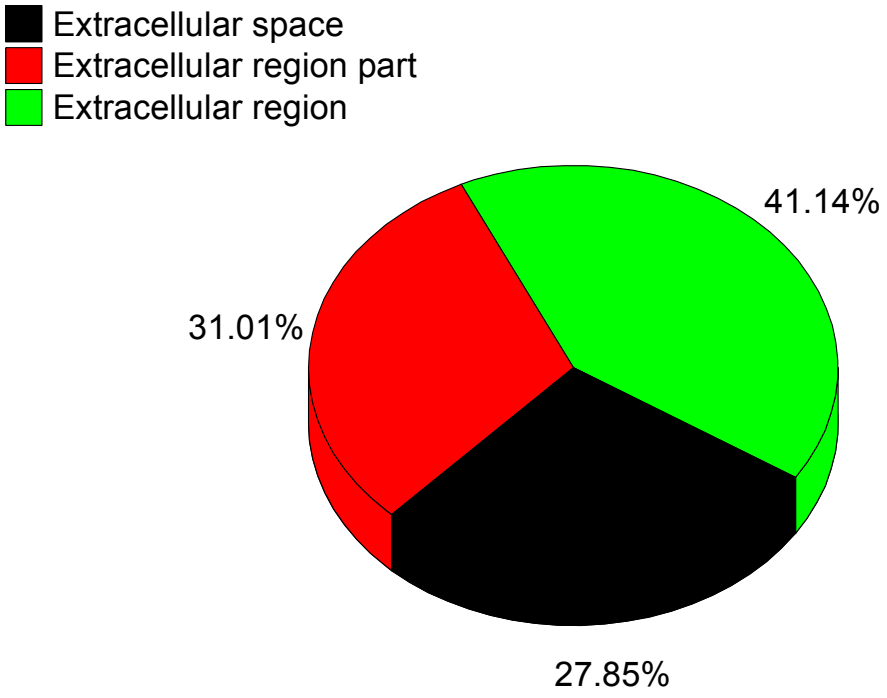
Figure 3-5.

Figure 3-6. Functional characterization of 513 genes down-regulated in corpora allata tissue after ETHR-RNAi. GO terms enriched as a) biological process b) cellular components and c) molecular function with the percentage of genes included in each term (Bonferroni test for multiple testing at $p<0.05$). Pie charts were built using Origin 8.1.

a)


Multicellular organism reproduction
Reproduction
Post-mating behavior
Polytene chromosome puffing
Heat shock-mediated polytene chromosome
Sperm competition
43.37%
5.42%
3.01%
3.01%
3.61%
41.57%

b)


Extracellular space
Extracellular region part
Extracellular region
41.14%
31.01%
27.85%

c)

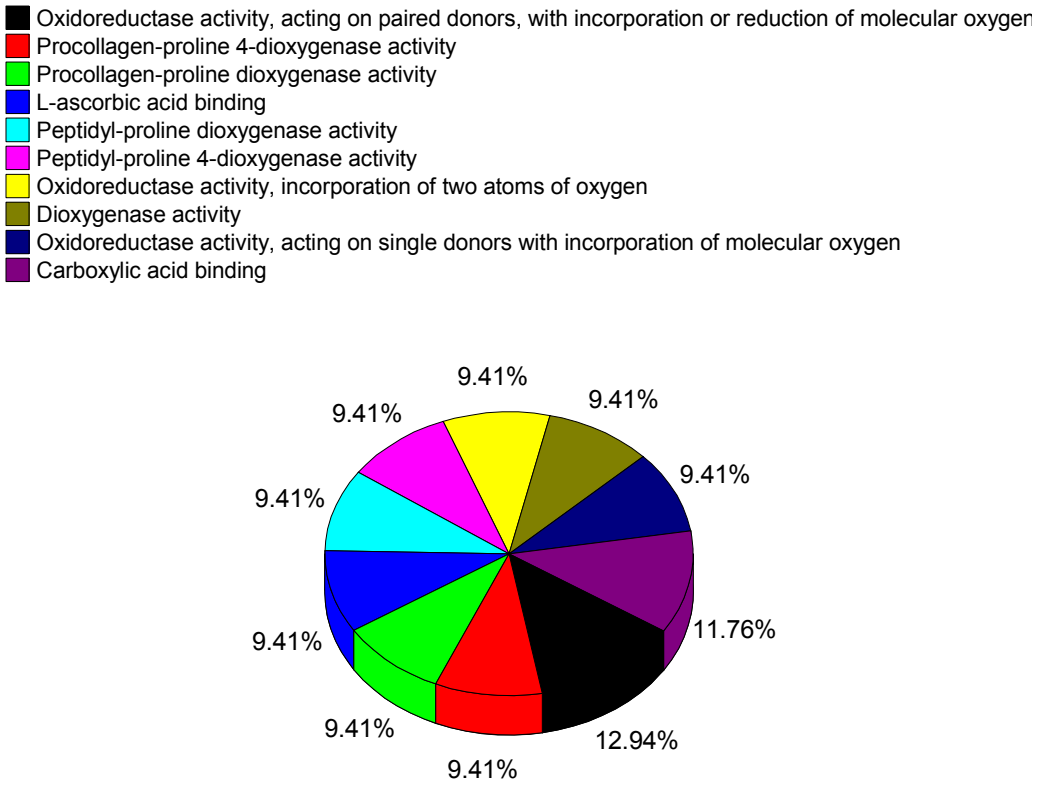

Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
Procollagen-proline 4-dioxygenase activity
Procollagen-proline dioxygenase activity
L-ascorbic acid binding
Peptidyl-proline dioxygenase activity
Peptidyl-proline 4-dioxygenase activity
Oxidoreductase activity, incorporation of two atoms of oxygen
Dioxygenase activity
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen
Carboxylic acid binding
9.41%
9.41%
9.41%
9.41%
9.41%
11.76%
9.41%
9.41%
12.94%
9.41%

Figure 3-6.

Figure 3-7. Fold change range of differentially expressed genes from head sample.

Number of genes plotted against the fold change range.

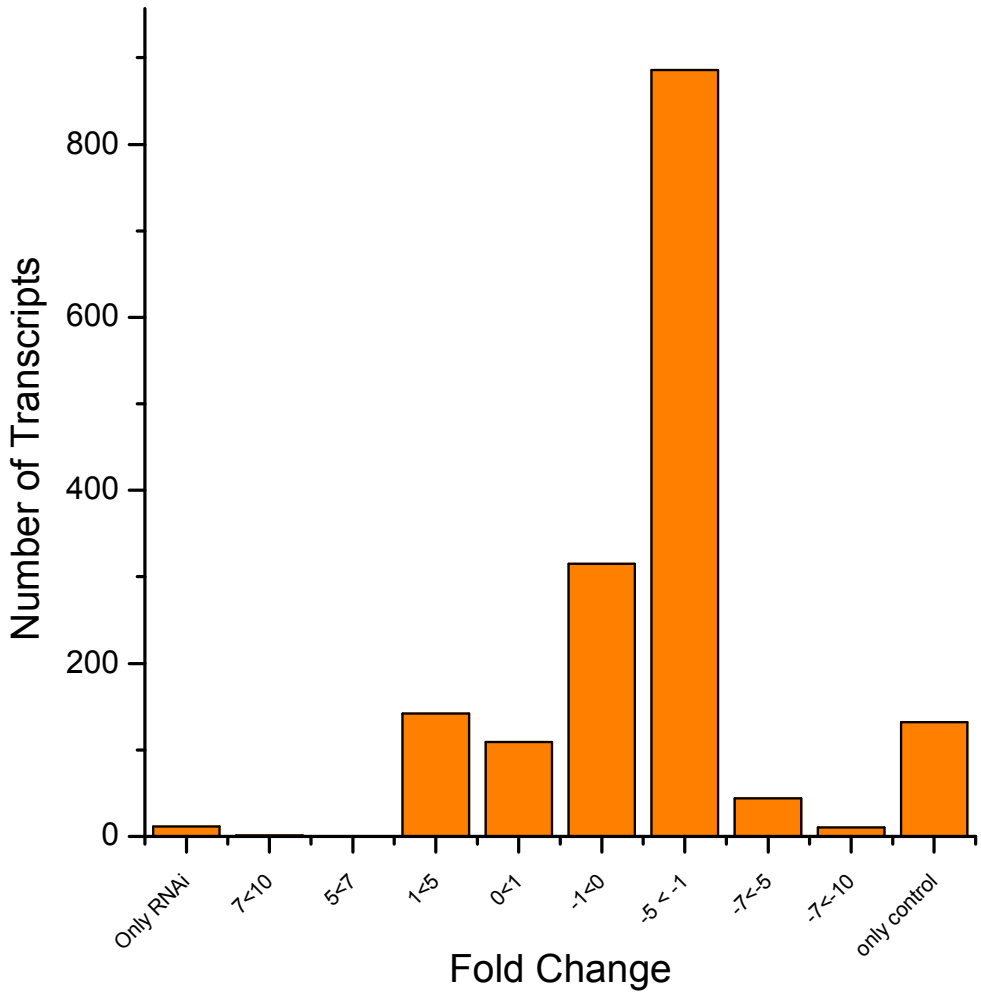

Number of Transcripts
800
600
400
200
0
Only RNAi
7<10
5<7
1<5
0<1
-1<0
-5 < -1
-7<-5
-7<-10
only control
Fold Change

Figure 3-7.

Figure 3-8. Chromosomal locations of differentially expressed genes from head sample. L indicates left arm, R indicates right arm, numbers indicate chromosomal number and U indicates unidentified. Up-regulated genes are represented in black and down-regulated in red.

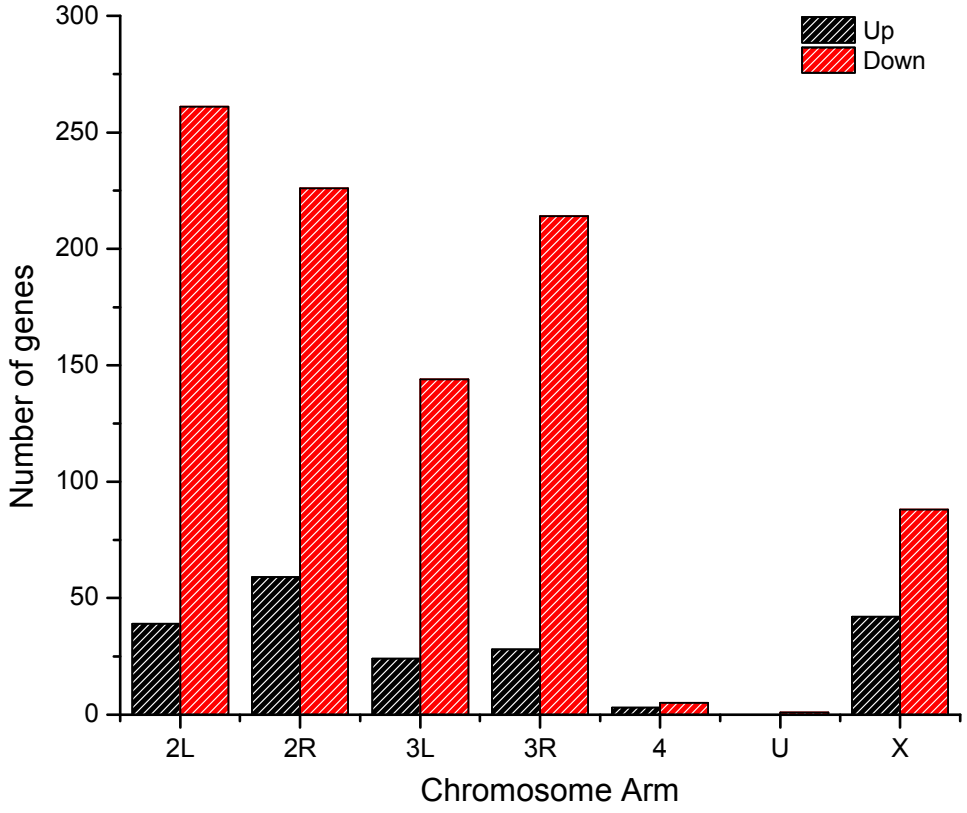

Up
Down
Number of genes
300
250
200
150
100
50
0
2L
2R
3L
3R
4
U
X
Chromosome Arm

Figure 3-8.

Figure 3-9. Tissue distribution levels of genes expressed significantly higher according to FlyAtlas from head sample. Up-regulated genes represented in green and down-regulated in yellow. MAG: male accessory glands.

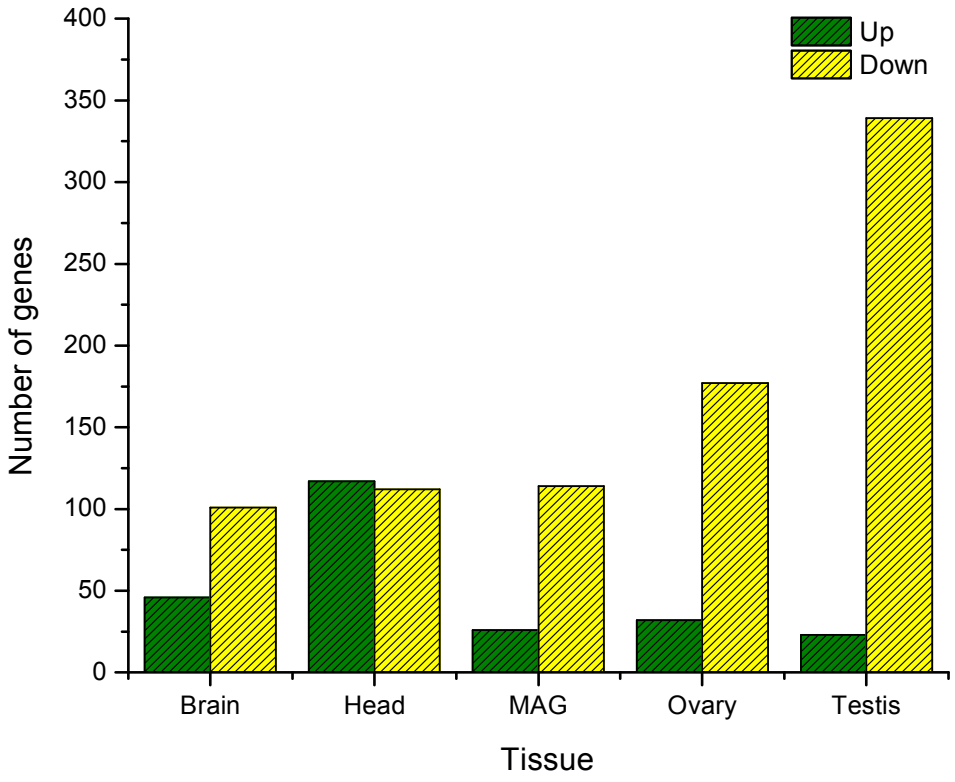

Up
Down
Number of genes
400
350
300
250
200
150
100
50
0
Brain
Head
MAG
Ovary
Testis
Tissue

Figure 3-9.

Figure 3-10. Functional characterization of 203 genes up-regulated in head sample after ETHR-RNAi. GO terms enriched as biological process with the percentage of genes included in each term (Bonferroni test for multiple testing at $p<0.05$). Pie charts were built using Origin 8.1.

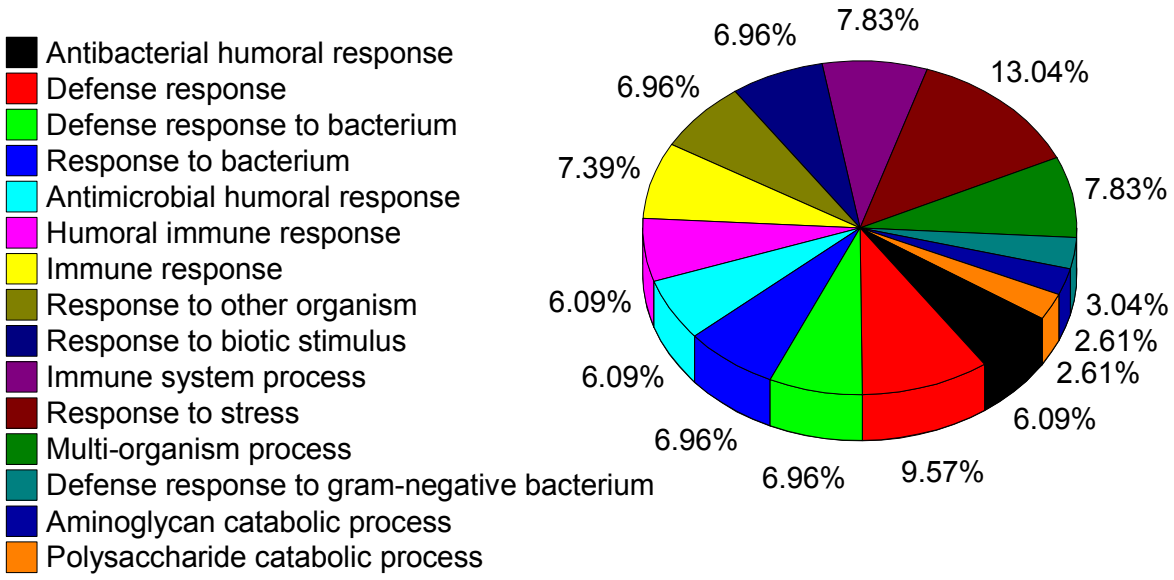
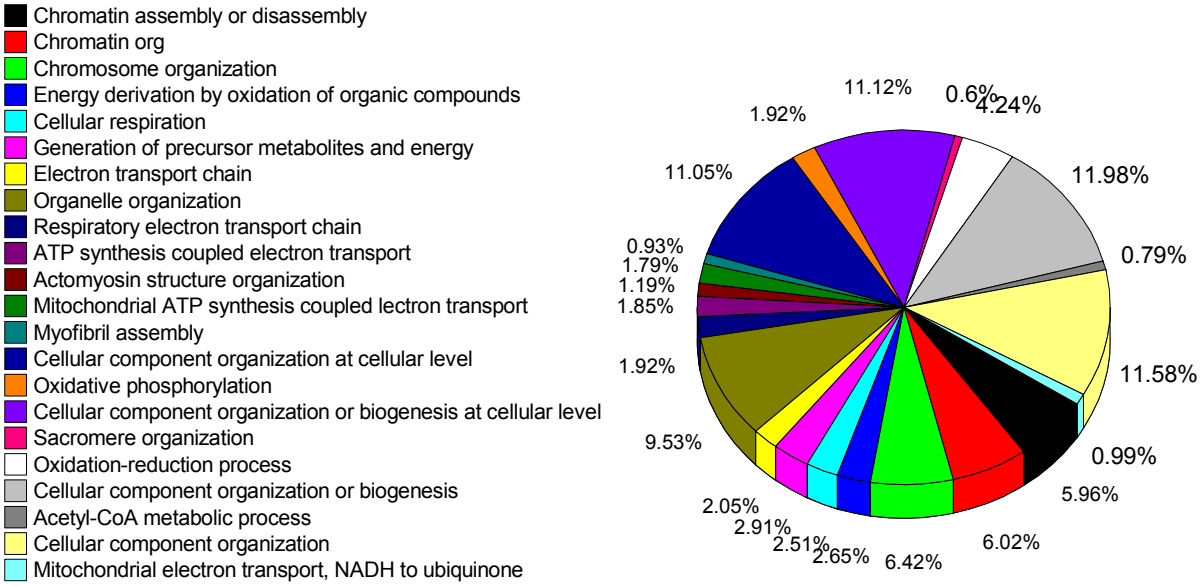

Antibacterial humoral response
Defense response
Defense response to bacterium
Response to bacterium
Antimicrobial humoral response
Humoral immune response
Immune response
Response to other organism
Response to biotic stimulus
Immune system process
Response to stress
Multi-organism process
Defense response to gram-negative bacterium
Aminoglycan catabolic process
Polysaccharide catabolic process
7.83%
13.04%
7.83%
3.04%
2.61%
2.61%
6.09%
9.57%
6.96%
6.96%
6.09%
6.09%
7.39%
6.96%

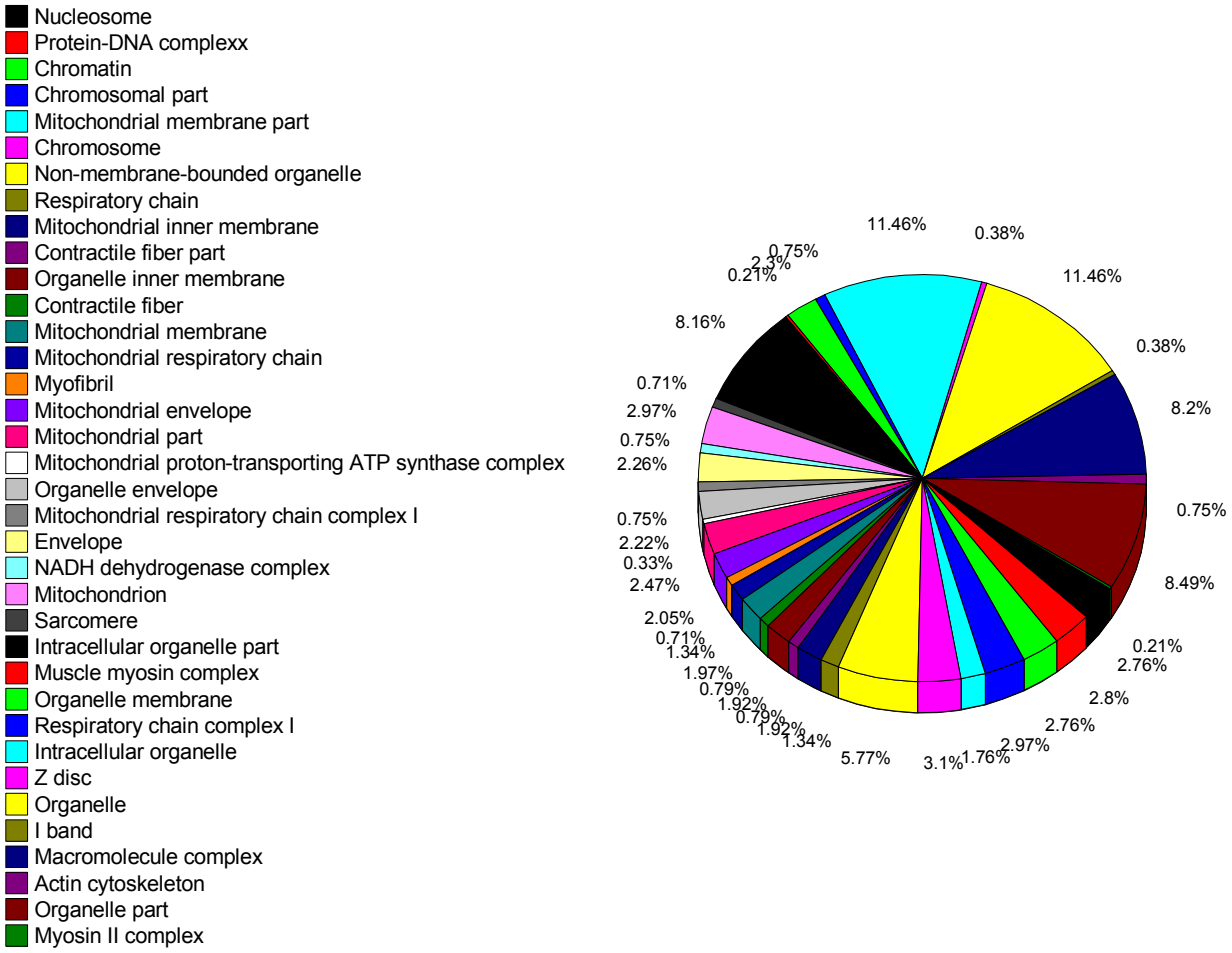
Figure 3-10.

Figure 3-11. Functional characterization of 954 genes down-regulated in head sample after ETHR-RNAi. GO terms enriched as a) biological process, b) cellular component and c) molecular function with the percentage of genes included in each term (Bonferroni test for multiple testing at $p<0.05$). Pie charts were built using Origin 8.1.

a)


Chromatin assembly or disassembly
Chromatin org
Chromosome organization
Energy derivation by oxidation of organic compounds
Cellular respiration
Generation of precursor metabolites and energy
Electron transport chain
Organelle organization
Respiratory electron transport chain
ATP synthesis coupled electron transport
Actomyosin structure organization
Mitochondrial ATP synthesis coupled lectron transport
Myofibril assembly
Cellular component organization at cellular level
Oxidative phosphorylation
Cellular component organization or biogenesis at cellular level
Sacromere organization
Oxidation-reduction process
Cellular component organization or biogenesis
Acetyl-CoA metabolic process
Cellular component organization
Mitochondrial electron transport, NADH to ubiquinone
11.12%
0.6%
4.24%
1.92%
11.05%
11.98%
0.93%
1.79%
1.19%
1.85%
0.79%
1.92%
11.58%
9.53%
0.99%
5.96%
2.05%
2.91%
2.51%
2.65%
6.42%
6.02%

b)


Nucleosome
Protein-DNA complexx
Chromatin
Chromosomal part
Mitochondrial membrane part
Chromosome
Non-membrane-bounded organelle
Respiratory chain
Mitochondrial inner membrane
Contractile fiber part
Organelle inner membrane
Contractile fiber
Mitochondrial membrane
Mitochondrial respiratory chain
Myofibril
Mitochondrial envelope
Mitochondrial part
Mitochondrial proton-transporting ATP synthase complex
Organelle envelope
Mitochondrial respiratory chain complex I
Envelope
NADH dehydrogenase complex
Mitochondrion
Sarcomere
Intracellular organelle part
Muscle myosin complex
Organelle membrane
Respiratory chain complex I
Intracellular organelle
Z disc
Organelle
I band
Macromolecule complex
Actin cytoskeleton
Organelle part
Myosin II complex
11.46%
0.38%
11.46%
0.38%
8.2%
0.75%
8.49%
0.21%
2.76%
2.8%
2.76%
2.97%
1.76%
3.1%
5.77%
1.34%
1.92%
0.79%
1.92%
0.79%
1.97%
1.34%
0.71%
2.05%
2.47%
0.33%
2.22%
0.75%
2.26%
0.75%
2.97%
0.71%
8.16%
0.21%
2.3%
0.75%

c)

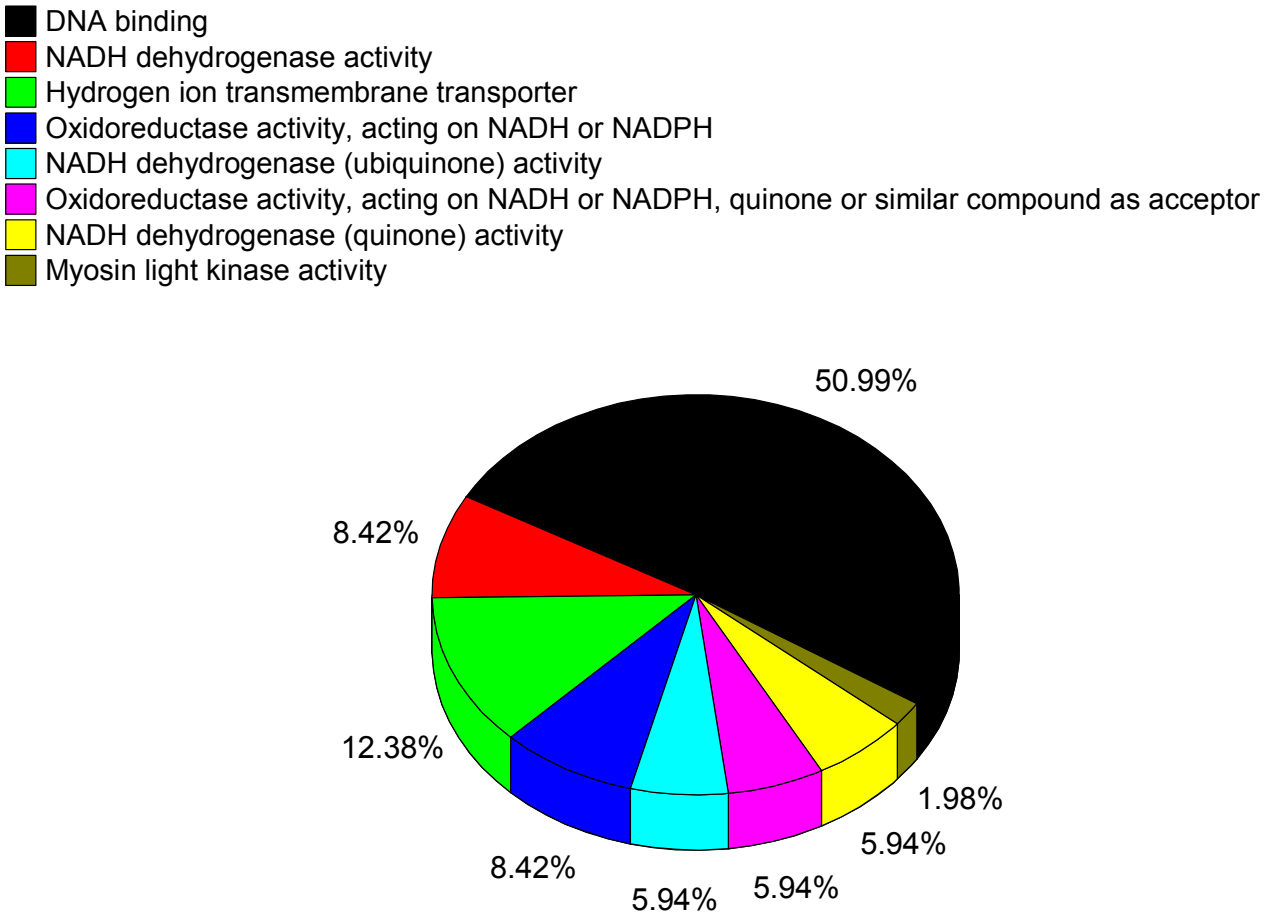

DNA binding
NADH dehydrogenase activity
Hydrogen ion transmembrane transporter
Oxidoreductase activity, acting on NADH or NADPH
NADH dehydrogenase (ubiquinone) activity
Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor
NADH dehydrogenase (quinone) activity
Myosin light kinase activity
50.99%
8.42%
12.38%
1.98%
5.94%
8.42%
5.94%
5.94%

Figure 3-11.

Figure 3-12. Fold change range of differentially expressed genes from whole fly sample.

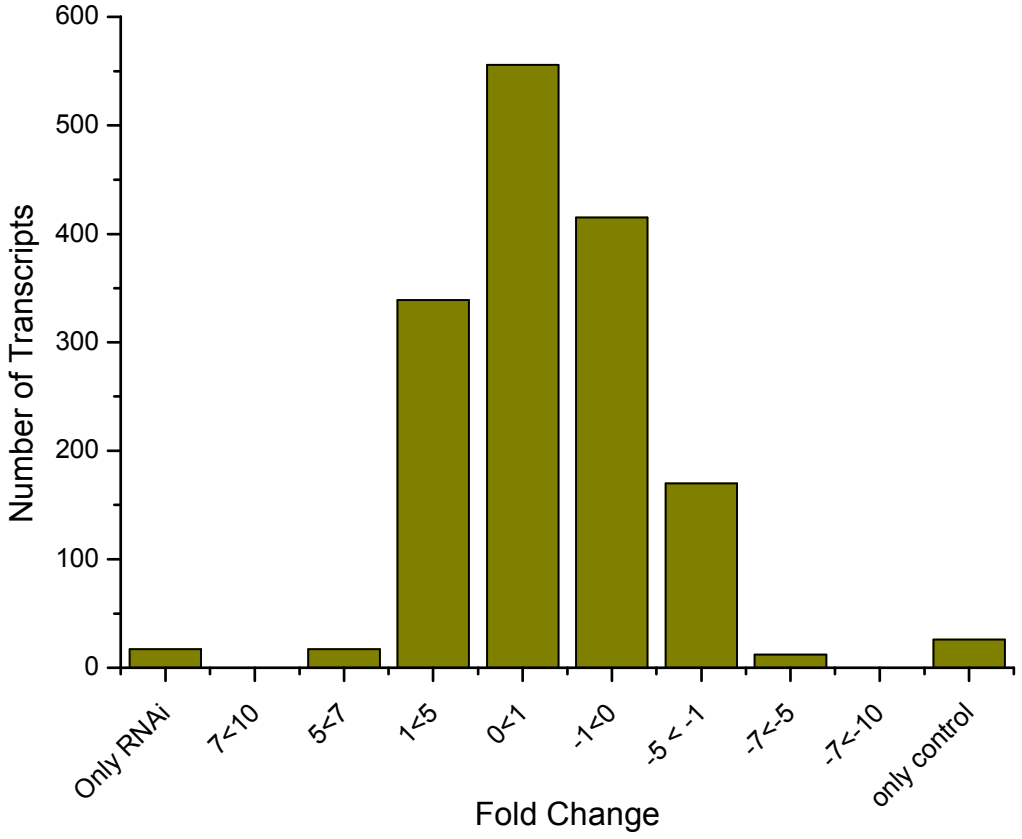

600
500
400
300
200
100
0
Number of Transcripts
Only RNAi
7<10
5<7
1<5
0<1
-1<0
-5 < -1
-7<-5
-7<-10
only control
Fold Change

Figure 3-12.

Figure 3-13. Chromosomal locations of differentially expressed genes from whole fly sample. L indicates left arm, R indicates right arm, numbers indicate chromosomal number and U indicates unidentified. Up-regulated genes are represented in black and down-regulated in red.

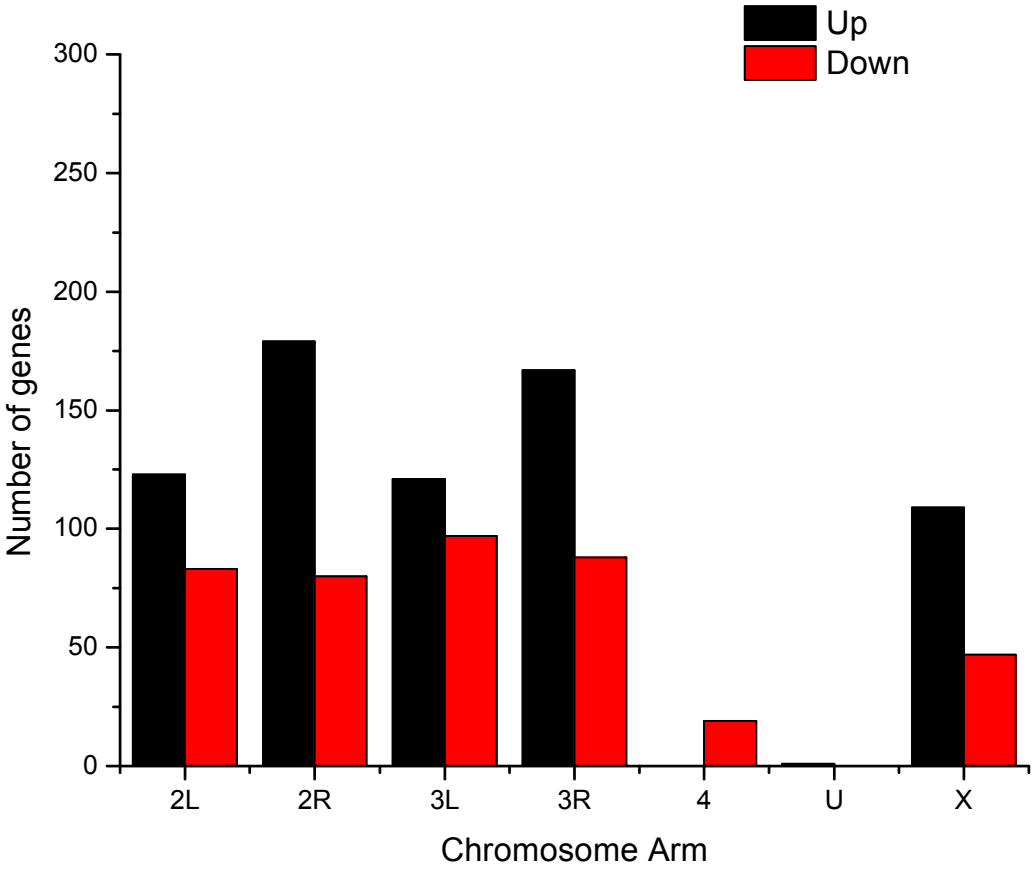

Up
Down
300
250
200
150
100
50
0
Number of genes
2L
2R
3L
3R
4
U
X
Chromosome Arm

Figure 3-13.

Figure 3-14. Tissue distribution levels of genes expressed significantly higher according to FlyAtlas from whole fly sample. Up-regulated genes represented in green and down-regulated in yellow. MAG: male accessory glands.

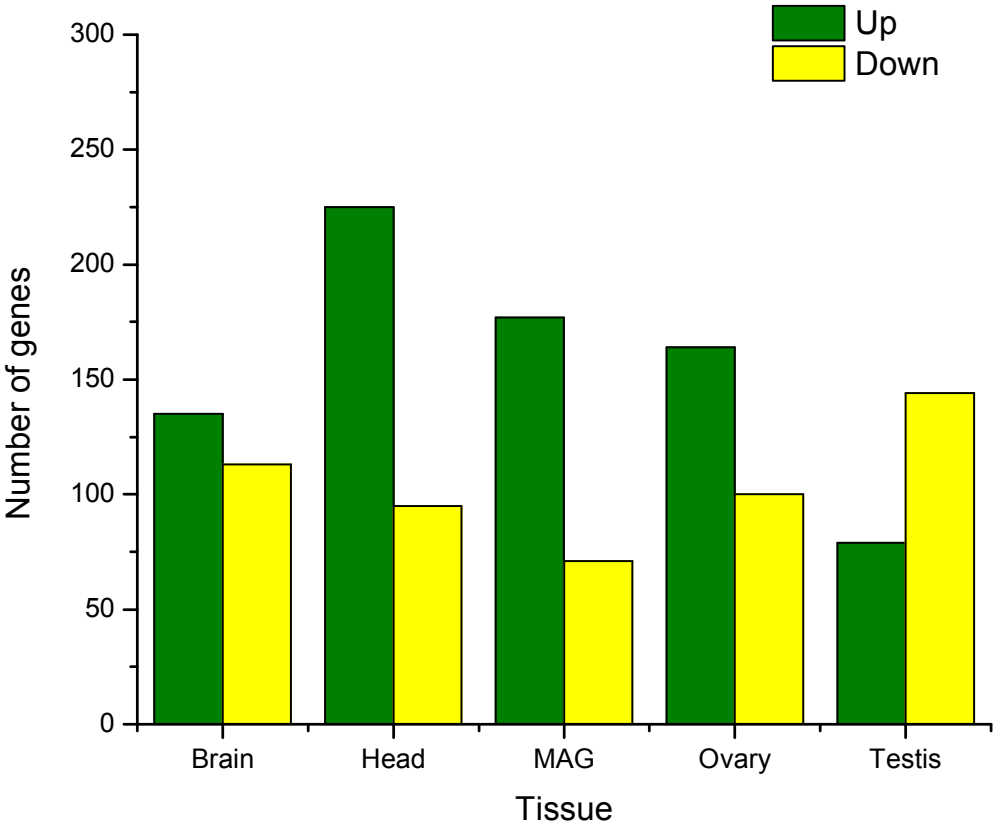
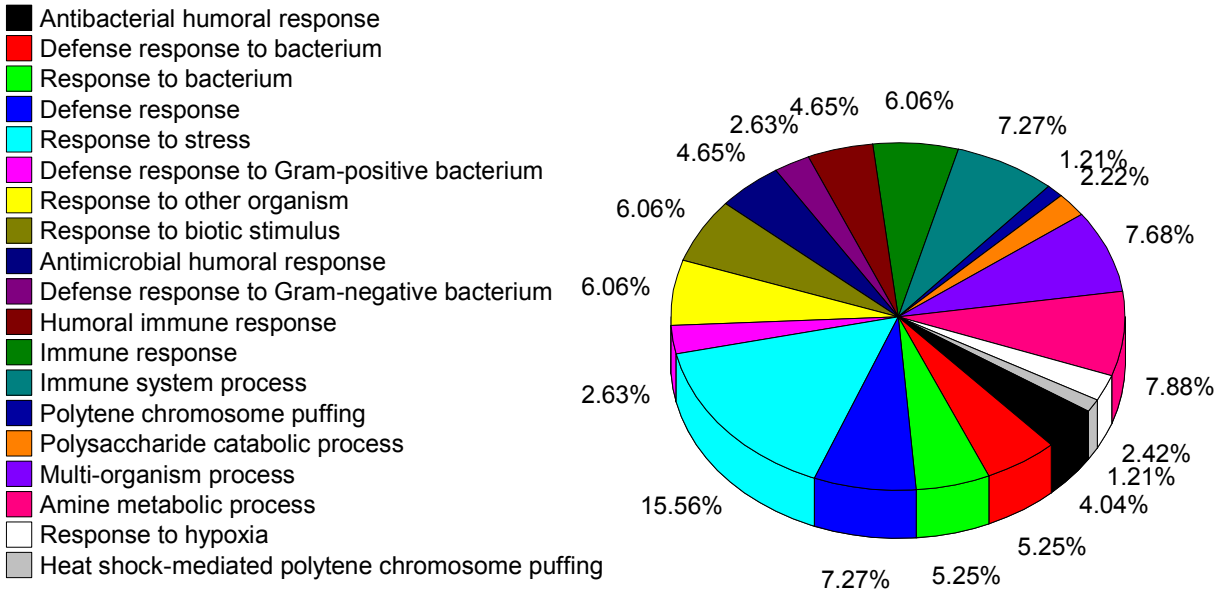

Up
Down
300
250
200
150
100
50
0
Number of genes
Brain
Head
MAG
Ovary
Testis
Tissue

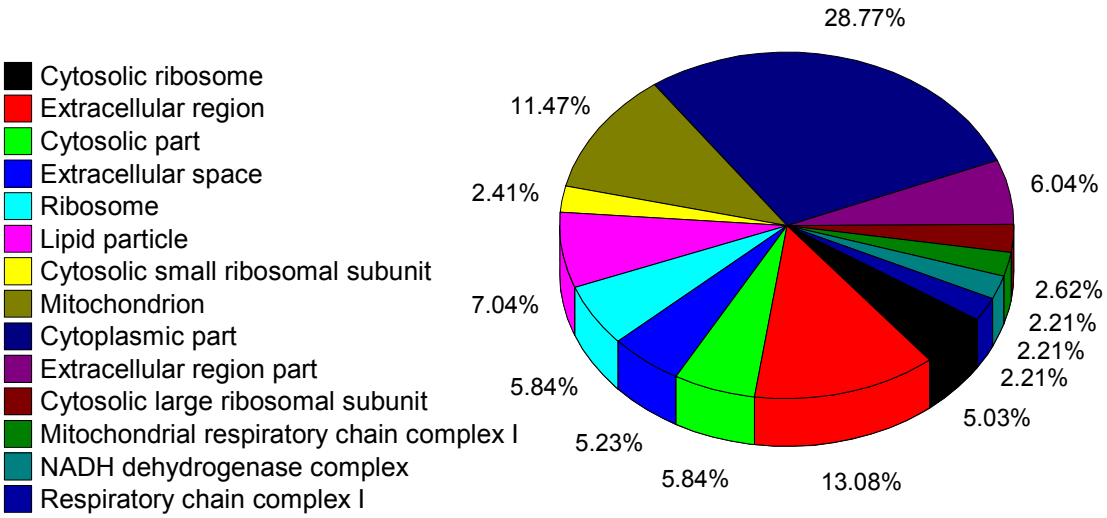
Figure 3-14.

Figure 3-15. Functional characterization of 729 genes up-regulated in whole fly sample after ETHR-RNAi. GO terms enriched as a) biological process, b) cellular component and c) molecular function with the percentage of genes included in each term (Bonferroni test for multiple testing at $p<0.05$). Pie charts were built using Origin 8.1.

a)


Antibacterial humoral response
Defense response to bacterium
Response to bacterium
Defense response
Response to stress
Defense response to Gram-positive bacterium
Response to other organism
Response to biotic stimulus
Antimicrobial humoral response
Defense response to Gram-negative bacterium
Humoral immune response
Immune response
Immune system process
Polytene chromosome puffing
Polysaccharide catabolic process
Multi-organism process
Amine metabolic process
Response to hypoxia
Heat shock-mediated polytene chromosome puffing
4.65%
6.06%
2.63%
7.27%
4.65%
1.21%
2.22%
6.06%
7.68%
6.06%
7.88%
2.63%
2.42%
1.21%
15.56%
4.04%
5.25%
7.27%
5.25%

b)


Cytosolic ribosome
Extracellular region
Cytosolic part
Extracellular space
Ribosome
Lipid particle
Cytosolic small ribosomal subunit
Mitochondrion
Cytoplasmic part
Extracellular region part
Cytosolic large ribosomal subunit
Mitochondrial respiratory chain complex I
NADH dehydrogenase complex
Respiratory chain complex I
28.77%
6.04%
2.62%
2.21%
2.21%
2.21%
5.03%
13.08%
5.84%
5.23%
5.84%
7.04%
2.41%
11.47%

c)

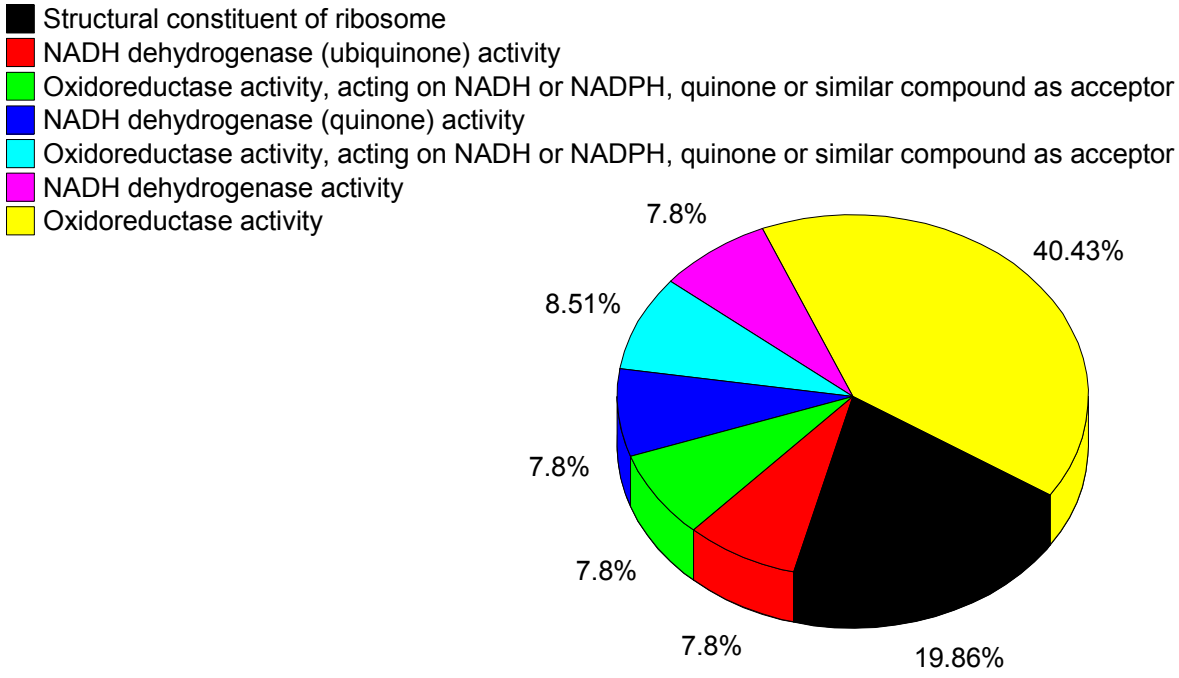

Structural constituent of ribosome
NADH dehydrogenase (ubiquinone) activity
Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor
NADH dehydrogenase (quinone) activity
Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor
NADH dehydrogenase activity
Oxidoreductase activity
7.8%
40.43%
8.51%
7.8%
7.8%
7.8%
19.86%

Figure 3-15.

Figure 3-16. Relative tissue distribution levels of genes expressed significantly higher according to FlyAtlas from three fly tissues. Up-regulated genes represented in green and down-regulated in yellow.

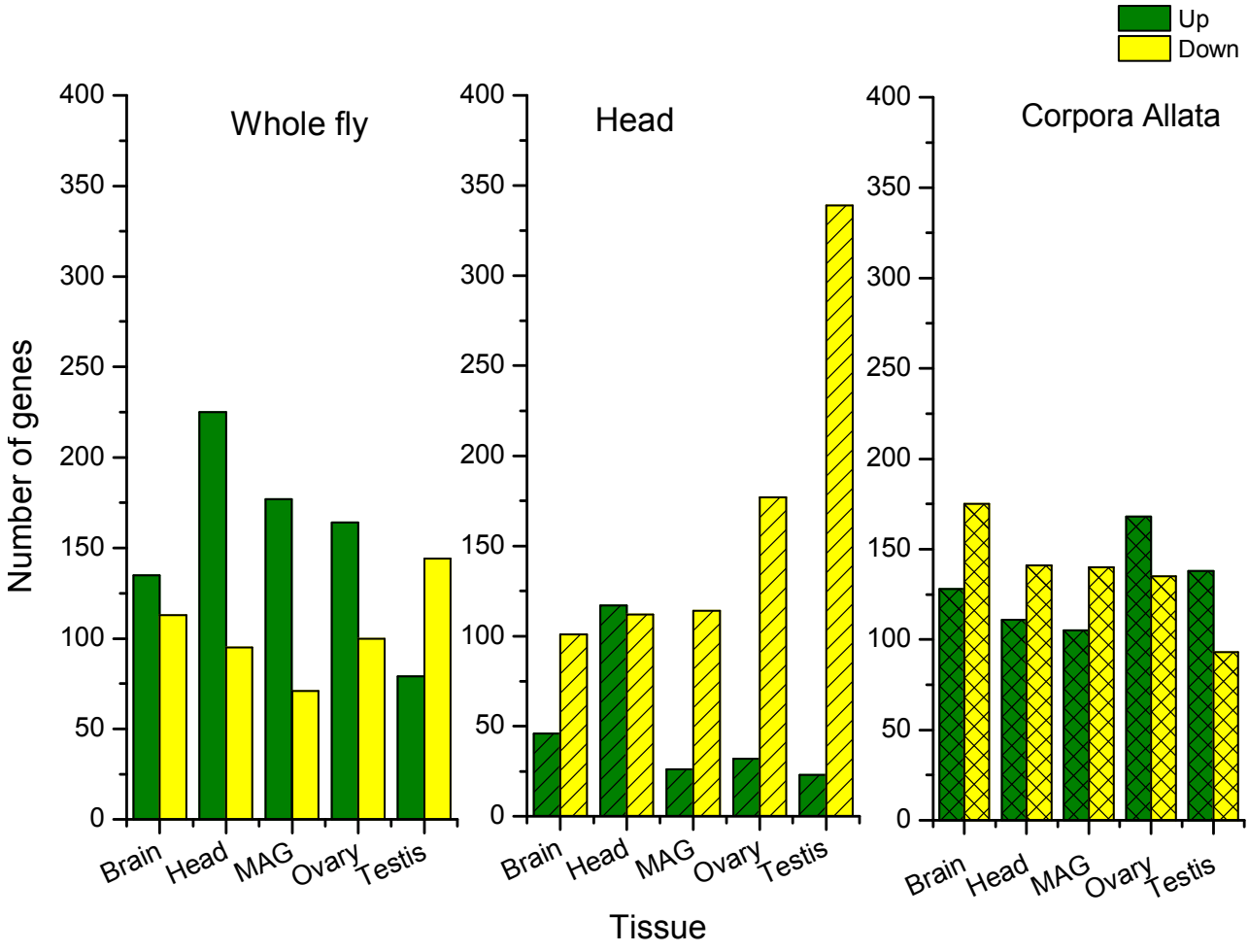
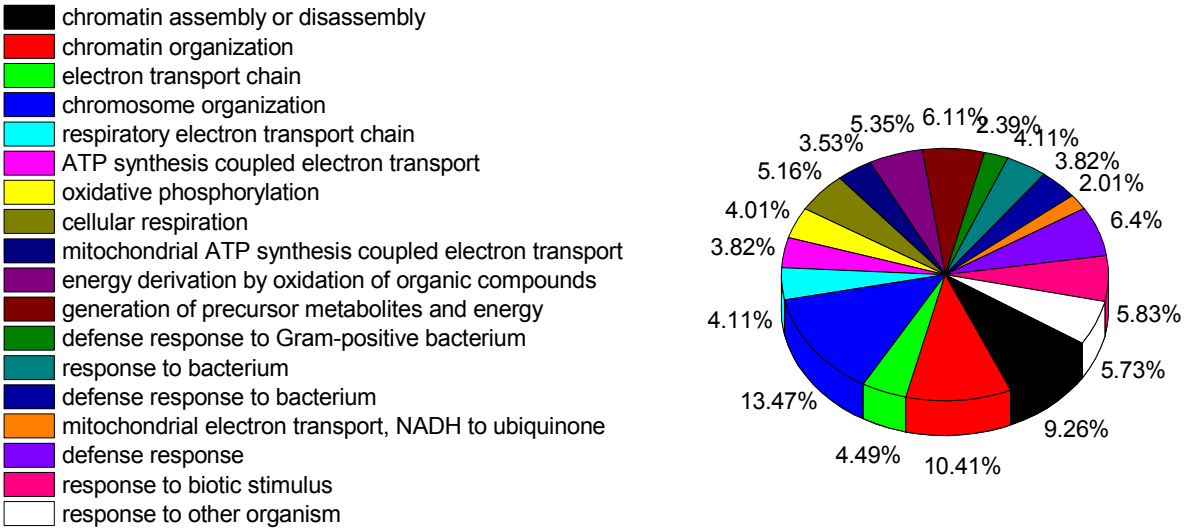

Up
Down
Whole fly
Head
Corpora Allata
Number of genes
0
50
100
150
200
250
300
350
400
Brain
Head
MAG
Ovary
Testis
Tissue

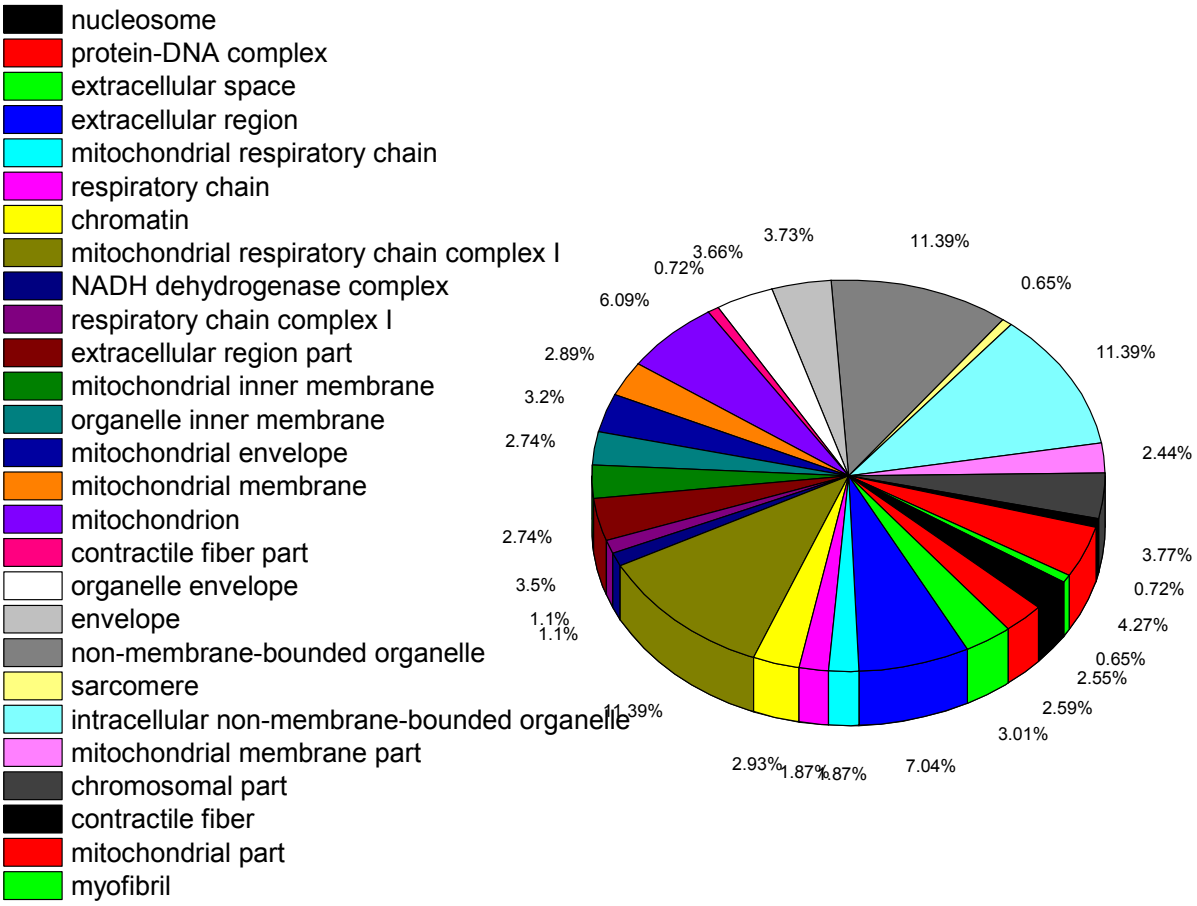
Figure 3-16.

Figure 3-17. Functional characterization of 2901 genes differentially expressed in three tissues after ETHR-RNAi. GO terms enriched as a) biological process, b) cellular component and c) molecular function with the percentage of genes included in each term (Bonferroni test for multiple testing at $p<0.05$). Pie charts were built using Origin 8.1.

a)


chromatin assembly or disassembly
chromatin organization
electron transport chain
chromosome organization
respiratory electron transport chain
ATP synthesis coupled electron transport
oxidative phosphorylation
cellular respiration
mitochondrial ATP synthesis coupled electron transport
energy derivation by oxidation of organic compounds
generation of precursor metabolites and energy
defense response to Gram-positive bacterium
response to bacterium
defense response to bacterium
mitochondrial electron transport, NADH to ubiquinone
defense response
response to biotic stimulus
response to other organism
5.35%
6.11%
2.39%
4.11%
3.82%
2.01%
6.4%
5.83%
5.73%
9.26%
10.41%
4.49%
13.47%
4.11%
3.82%
4.01%
5.16%
3.53%

b)


nucleosome
protein-DNA complex
extracellular space
extracellular region
mitochondrial respiratory chain
respiratory chain
chromatin
mitochondrial respiratory chain complex I
NADH dehydrogenase complex
respiratory chain complex I
extracellular region part
mitochondrial inner membrane
organelle inner membrane
mitochondrial envelope
mitochondrial membrane
mitochondrion
contractile fiber part
organelle envelope
envelope
non-membrane-bounded organelle
sarcomere
intracellular non-membrane-bounded organelle
mitochondrial membrane part
chromosomal part
contractile fiber
mitochondrial part
myofibril
3.73%
11.39%
3.66%
0.72%
0.65%
6.09%
11.39%
2.89%
2.44%
3.2%
2.74%
3.77%
2.74%
0.72%
3.5%
4.27%
1.1%
1.1%
0.65%
2.55%
11.39%
2.59%
3.01%
2.93%
1.87%
1.87%
7.04%

c)

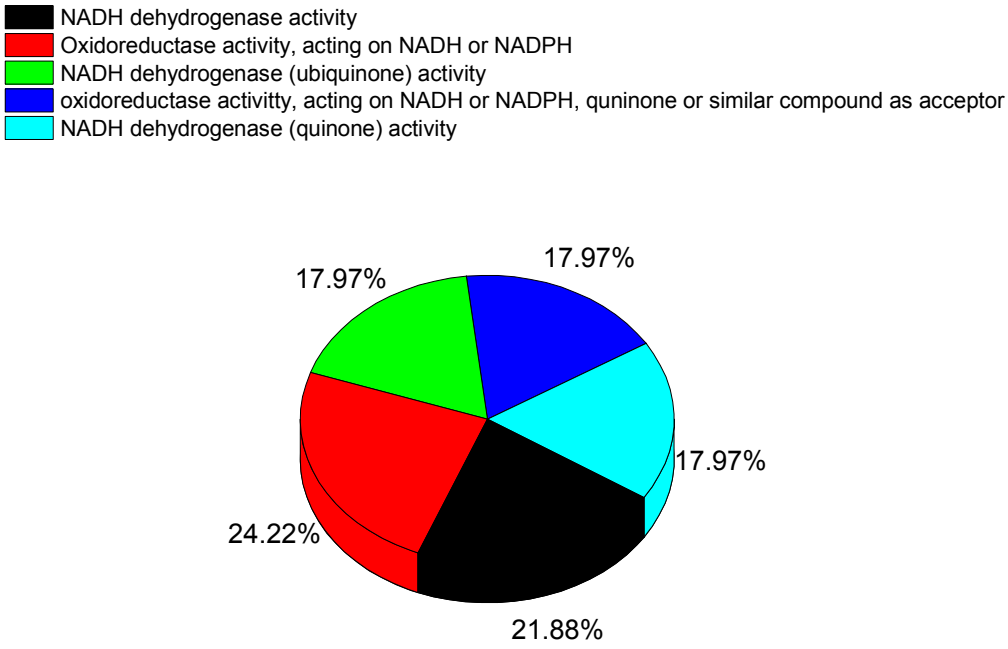

NADH dehydrogenase activity
Oxidoreductase activity, acting on NADH or NADPH
NADH dehydrogenase (ubiquinone) activity
oxidoreductase activitty, acting on NADH or NADPH, quninone or similar compound as acceptor
NADH dehydrogenase (quinone) activity
17.97%
17.97%
17.97%
24.22%
21.88%

Figure 3-17.

Figure 3-18. Quantitative PCR analysis showing relative fold change in tissues after ETHR-RNAi.

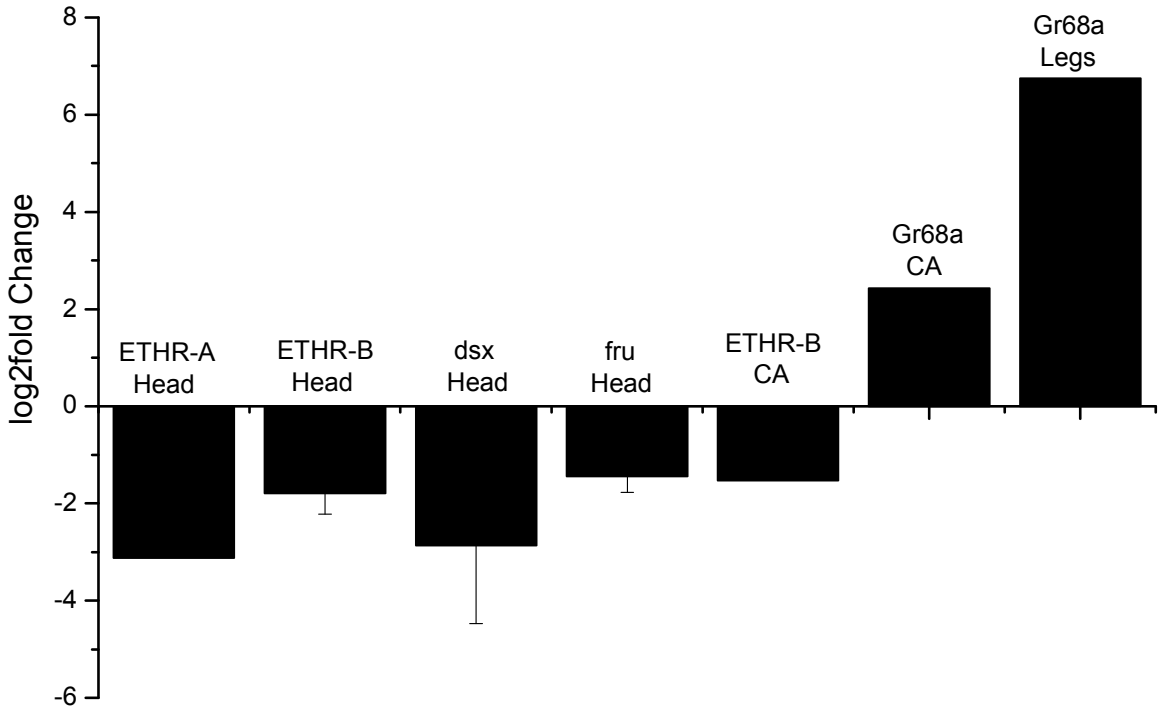

log2fold Change
8
6
4
2
0
-2
-4
-6
ETHR-A
Head
ETHR-B
Head
dsx
Head
fru
Head
ETHR-B
CA
Gr68a
CA
Gr68a
Legs

Figure 3-18.

Figure 3-19. Differentially expressed JH related genes. Blue boxes with up-arrow indicates up-regulated genes and red boxes with down-arrow indicates down-regulated genes. Figure modified from Huang et al, 2011.

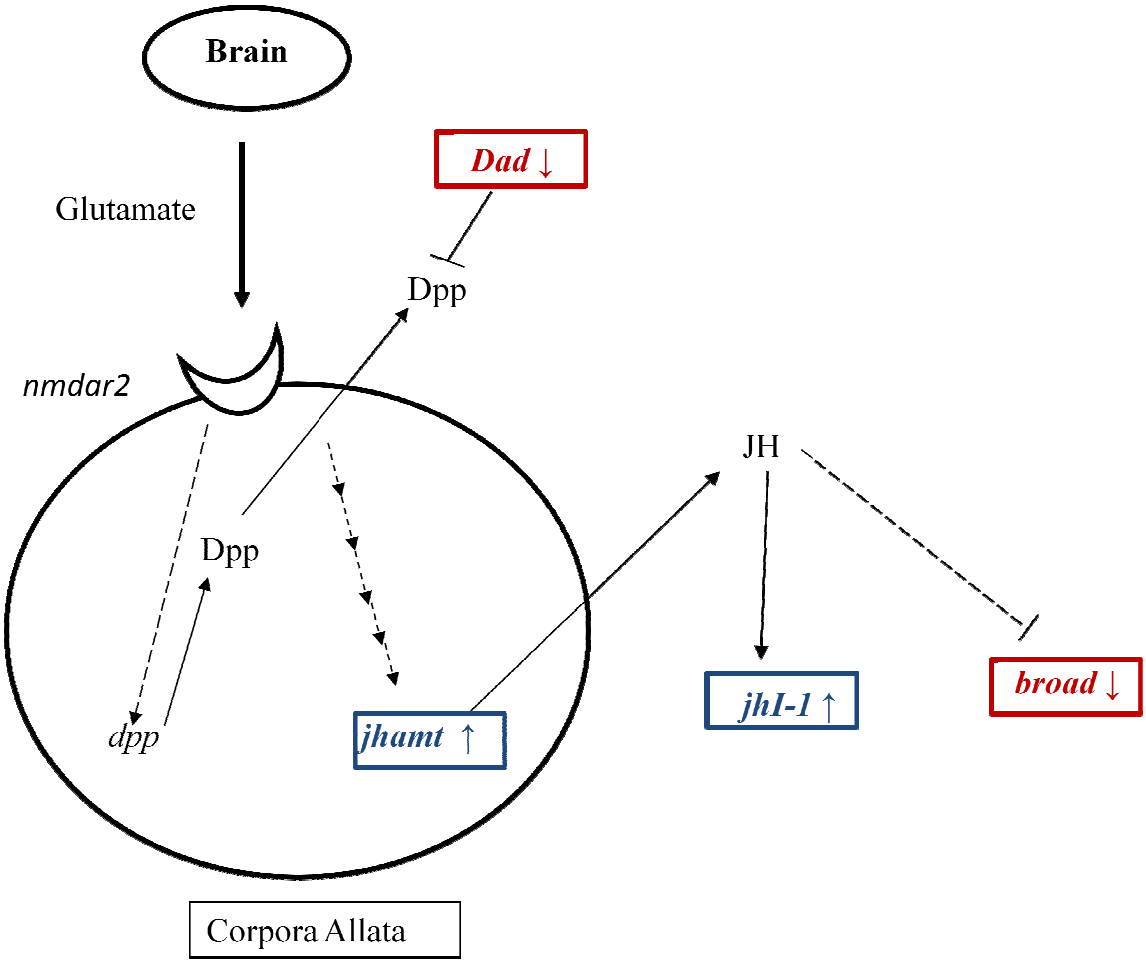

Brain
Glutamate
Dad ↓
Dpp
nmdar2
Dpp
dpp
jhamt ↑
JH
jhI-1 ↑
broad ↓
Corpora Allata

Figure 3-19.

Figure 3-20. Model explaining possible mechanisms involved under ETHR regulating male-male courtship.

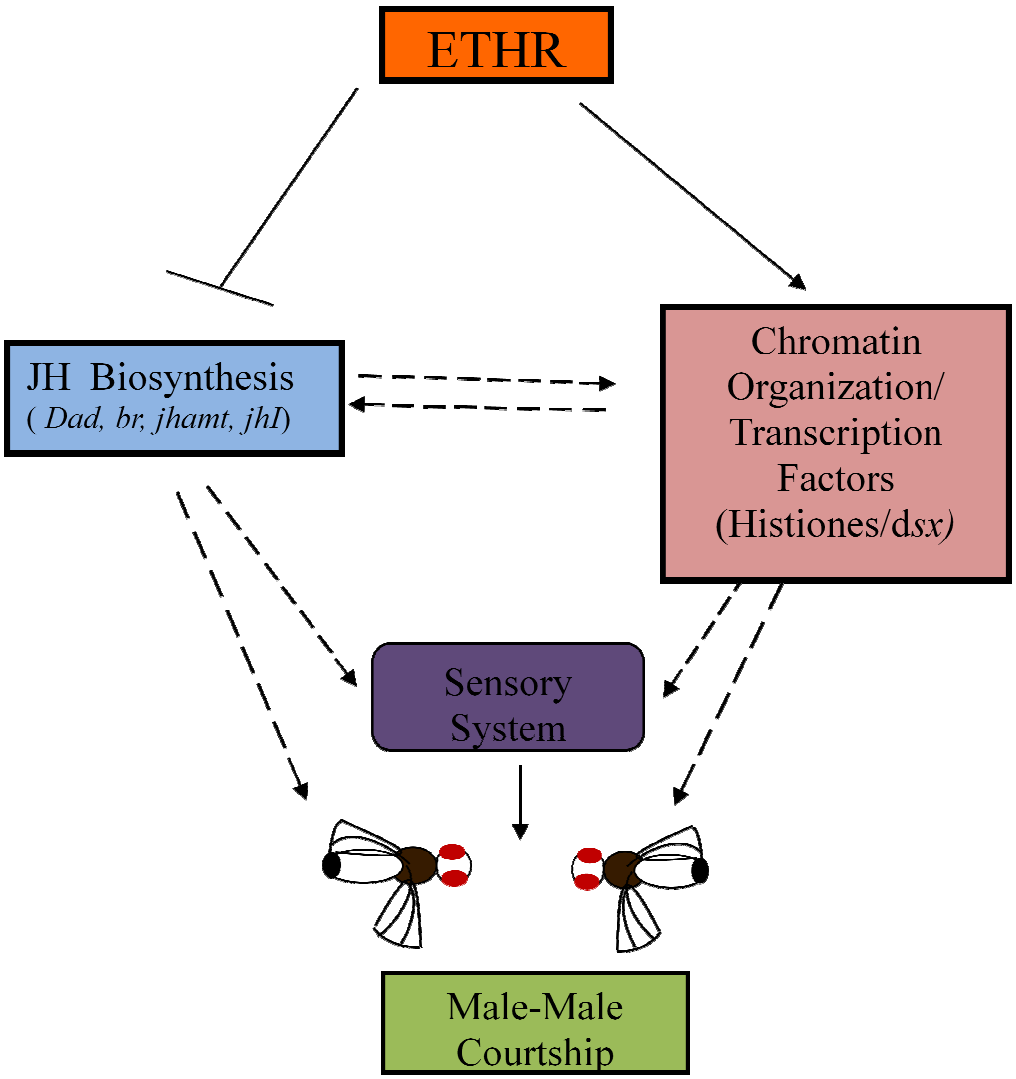

ETHR
JH Biosynthesis
(Dad, br, jhamt, jhI)
Chromatin
Organization/
Transcription
Factors
(Histiones/dsx)
Sensory
System
Male-Male
Courtship

Figure 3-20.

Figure 3-21. RNAseq control head sample showing reads for doublesex. Reads from female specific exon mapping from male head sample.

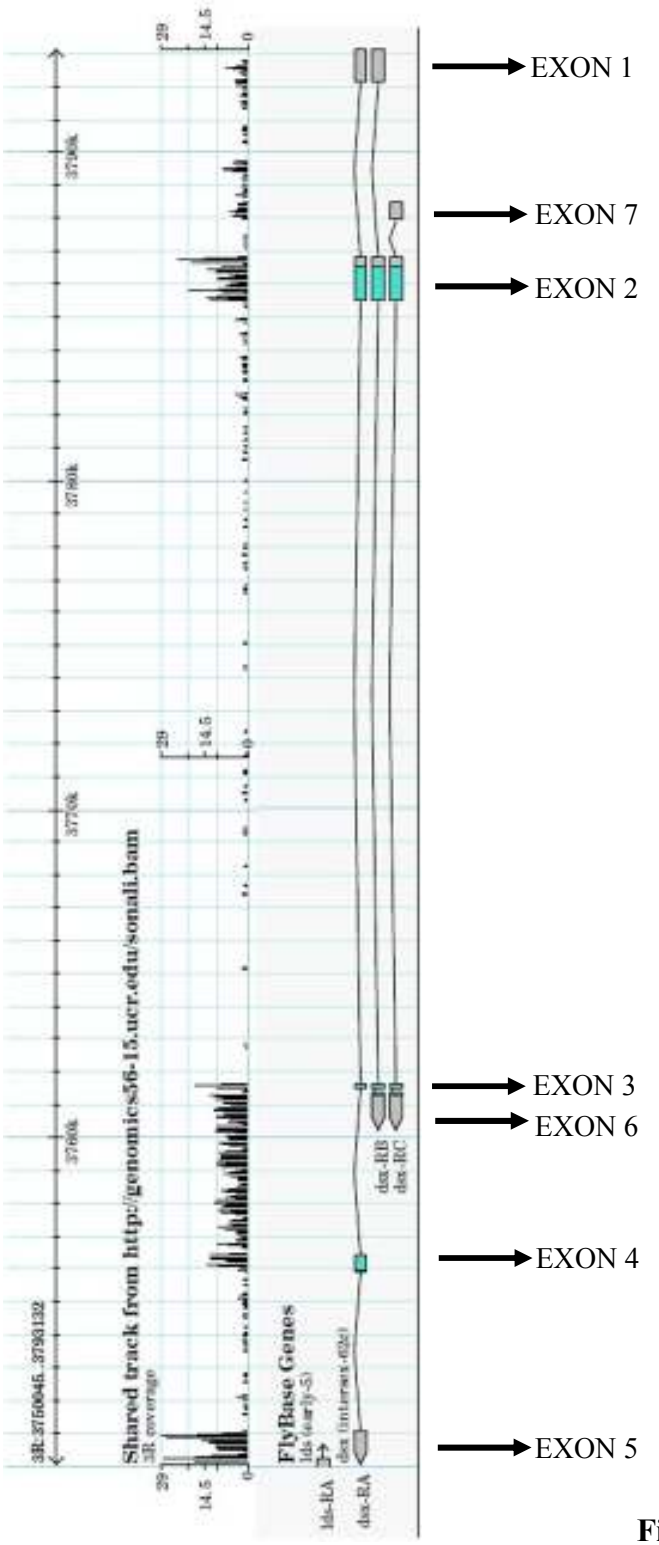

EXON 1
EXON 7
EXON 2
EXON 3
EXON 6
EXON 4
EXON 5
Shared track from http://genomics56-15.ucr.edu/sonali.bam
FlyBase Genes
3R:3760045..3793132
3R coverage
ids-RA
dsx-RA
dsx-RB
dsx-RC

Figure 3-21.

Figure 3-22. Doublesex genomic map with new exon. Exons in blue color are common in both males and females. Exons shown in green are specific for males, exons shown in red are female specific. Exon shown in orange (exon-8) is the new exon identified in males. Orange arrows represent PCR forward (frwd) and reverse (rev) primers.

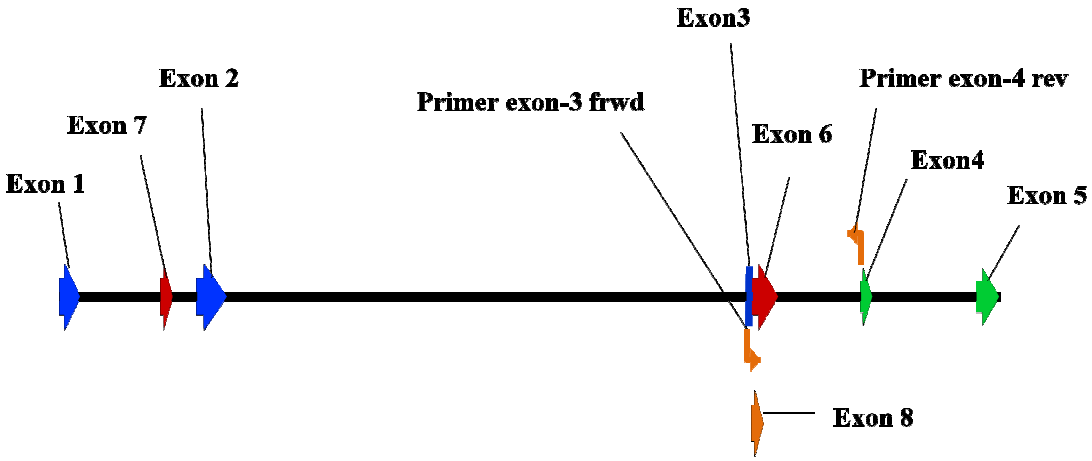

Exon3
Exon 2
Primer exon-3 frwd
Primer exon-4 rev
Exon 7
Exon 6
Exon4
Exon 1
Exon 5
Exon 8
Doublesex-Genomic

Figure 3-22.

Figure 3-23. Venn diagram showing number of differentially expressed genes from three samples.

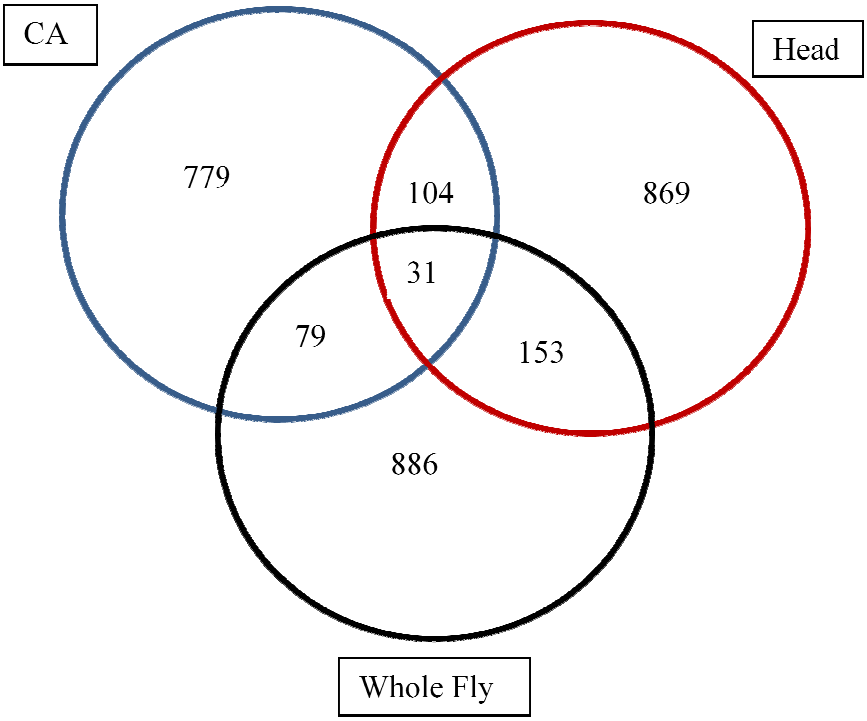
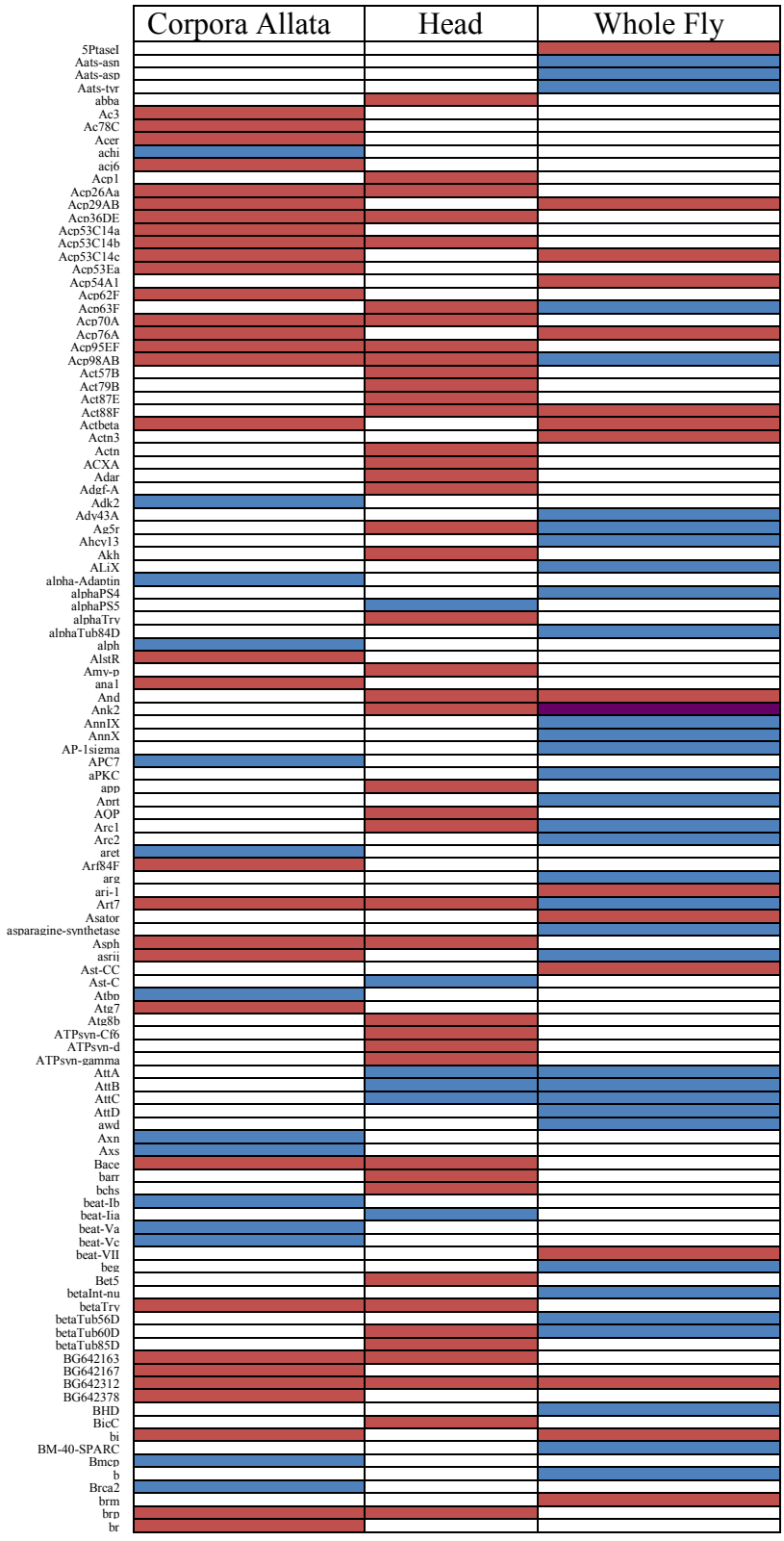


Figure 3-23.

Figure 3-24. Heatmap comparing Differentially Expressed Genes in CA, Head and Whole Flies. Blue: Up-regulated; Red: Down-regulated; White: No Change/Not Detected; Purple: Both Up-and Down-regulated.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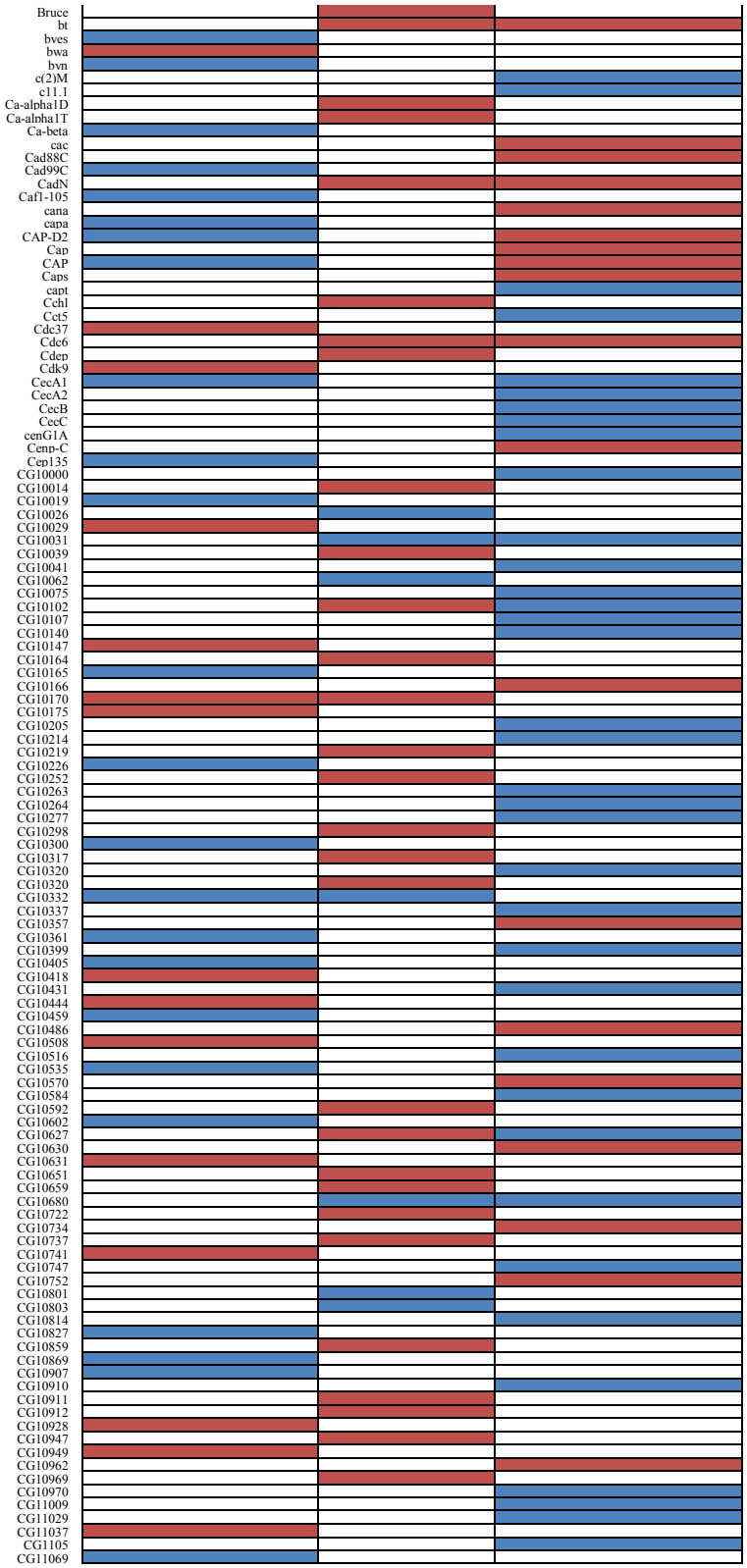
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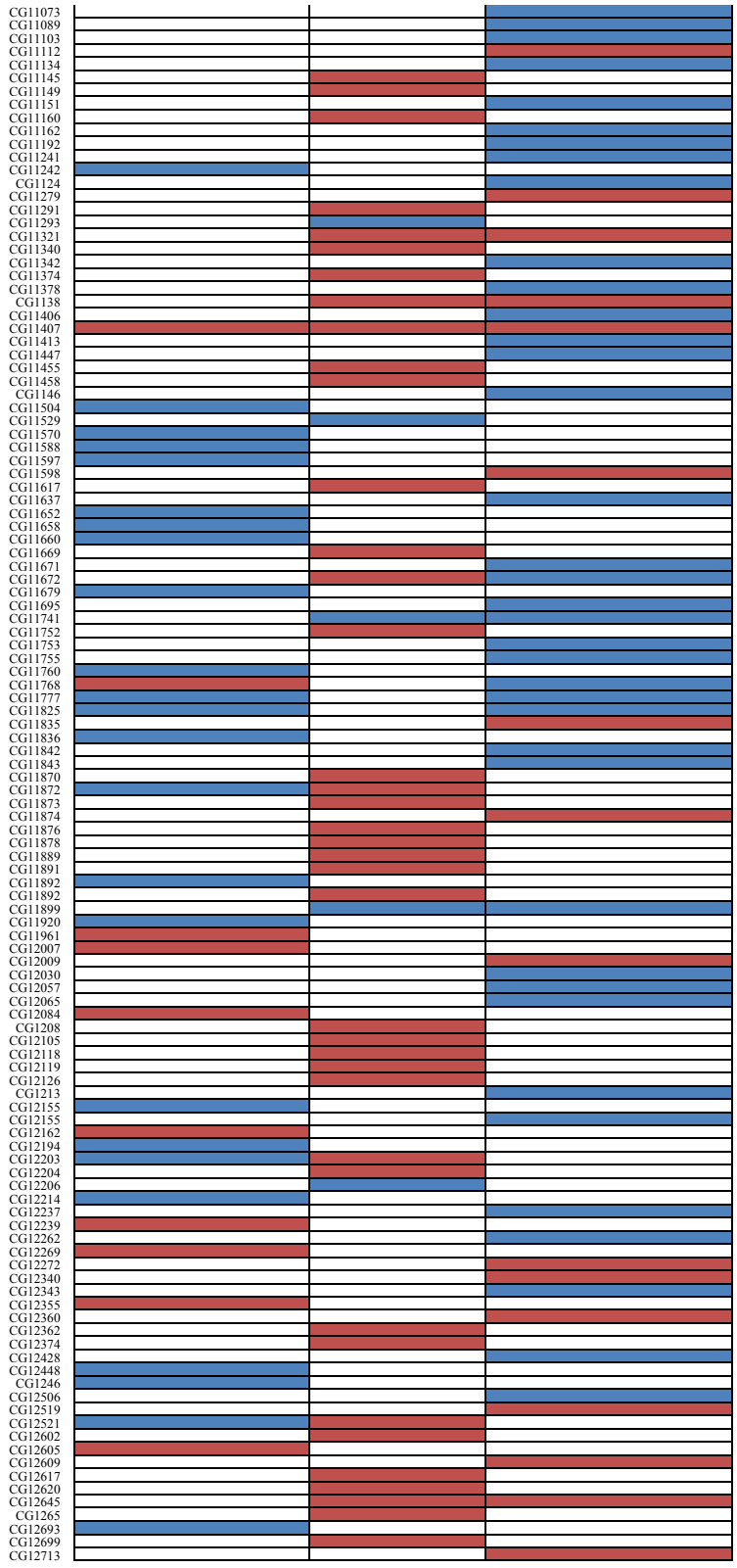
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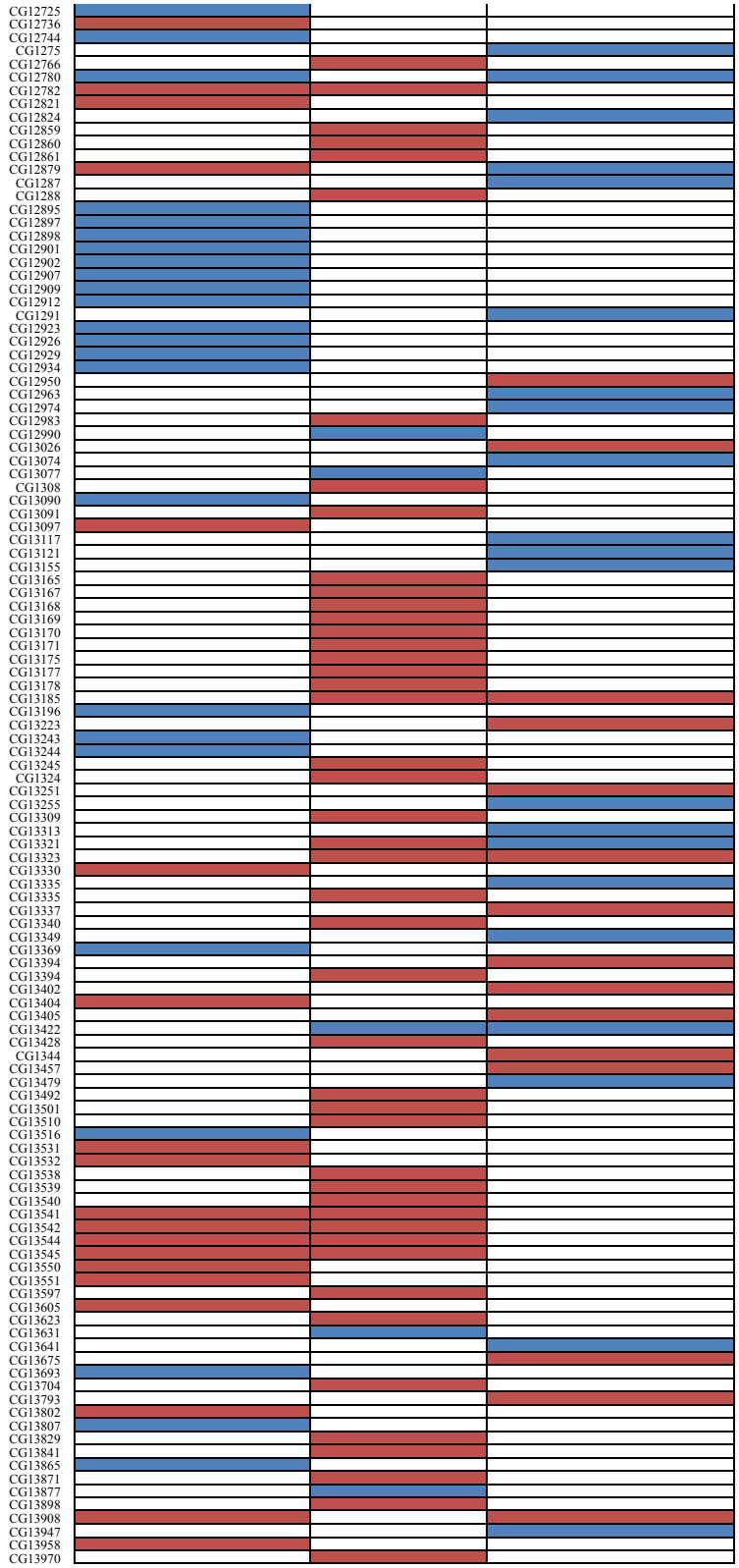
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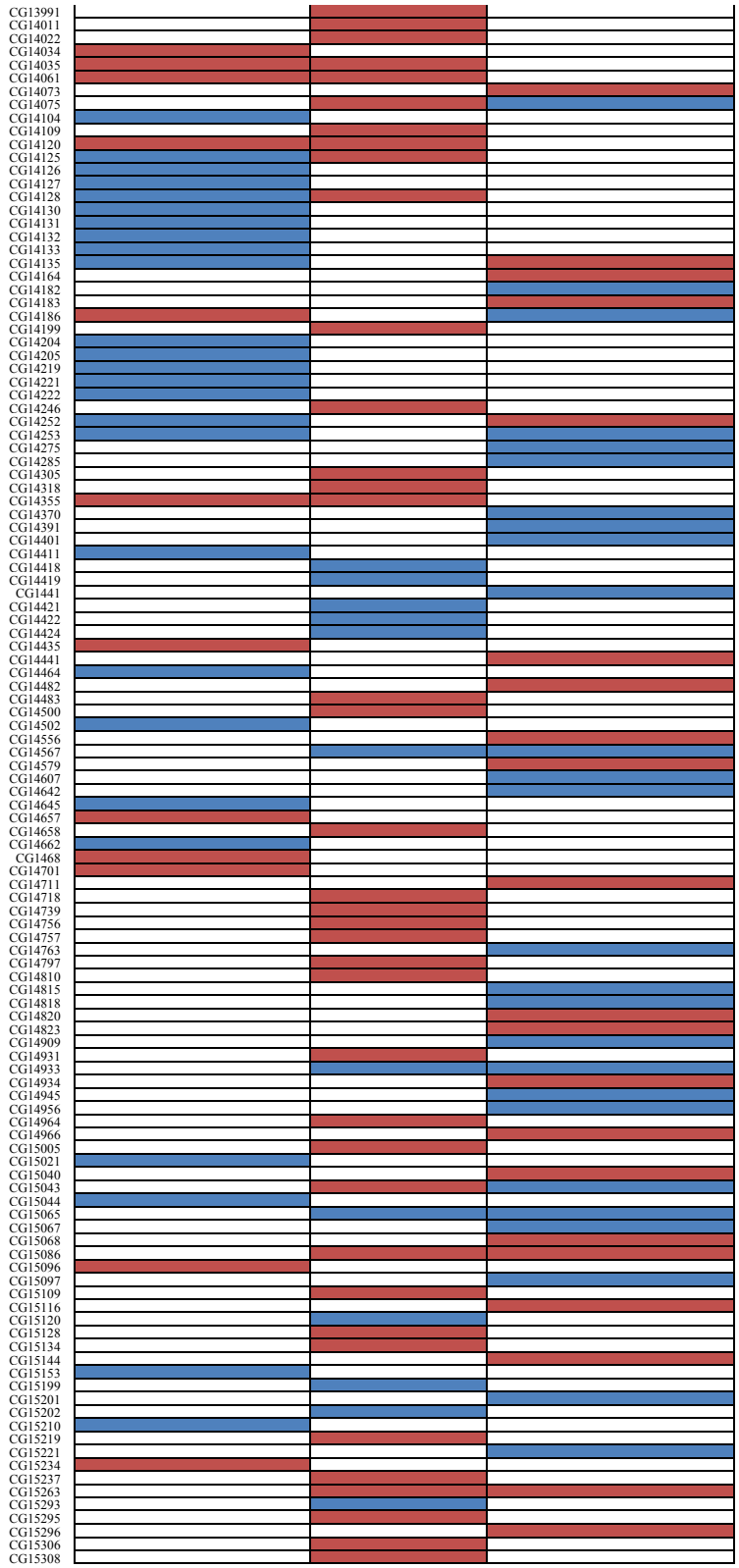
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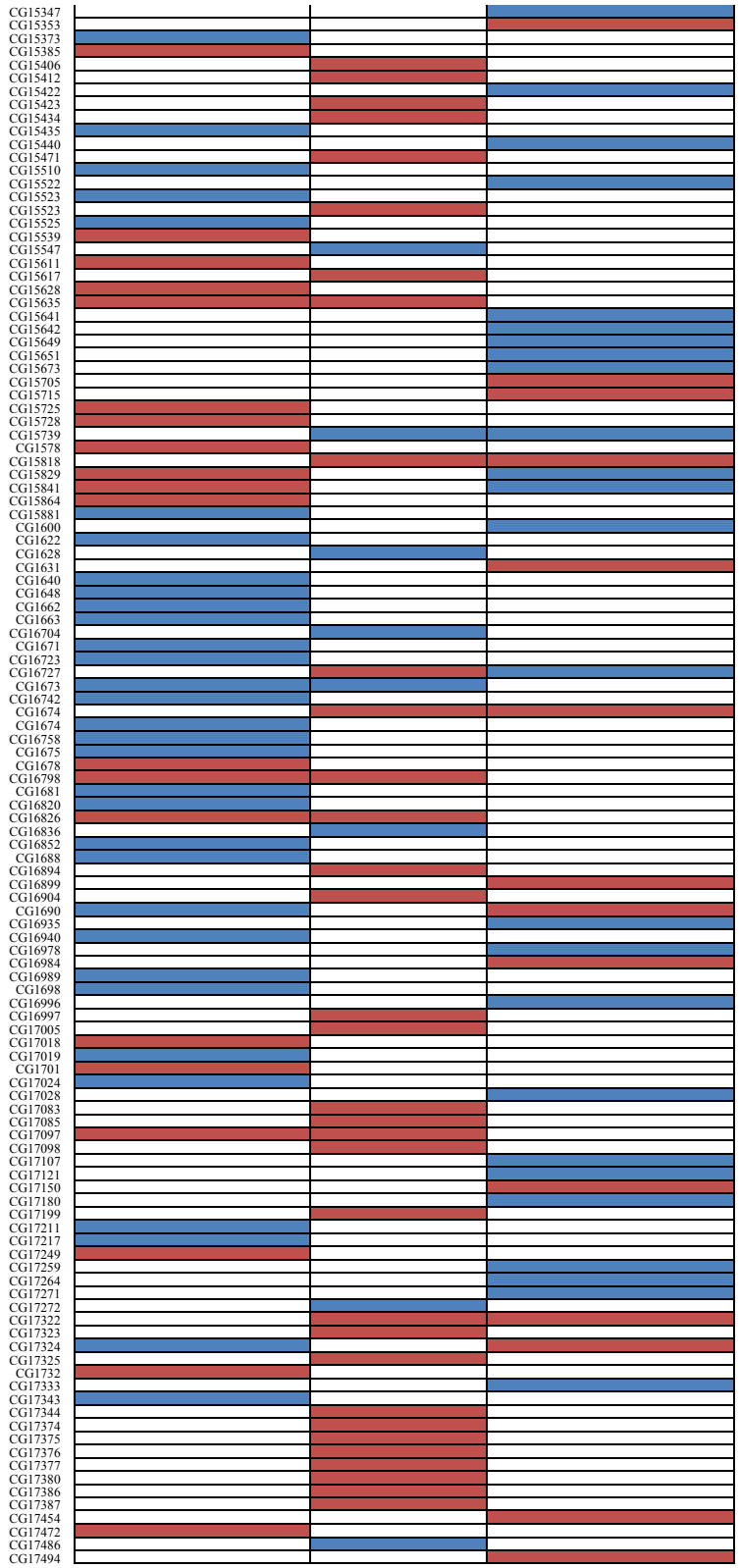
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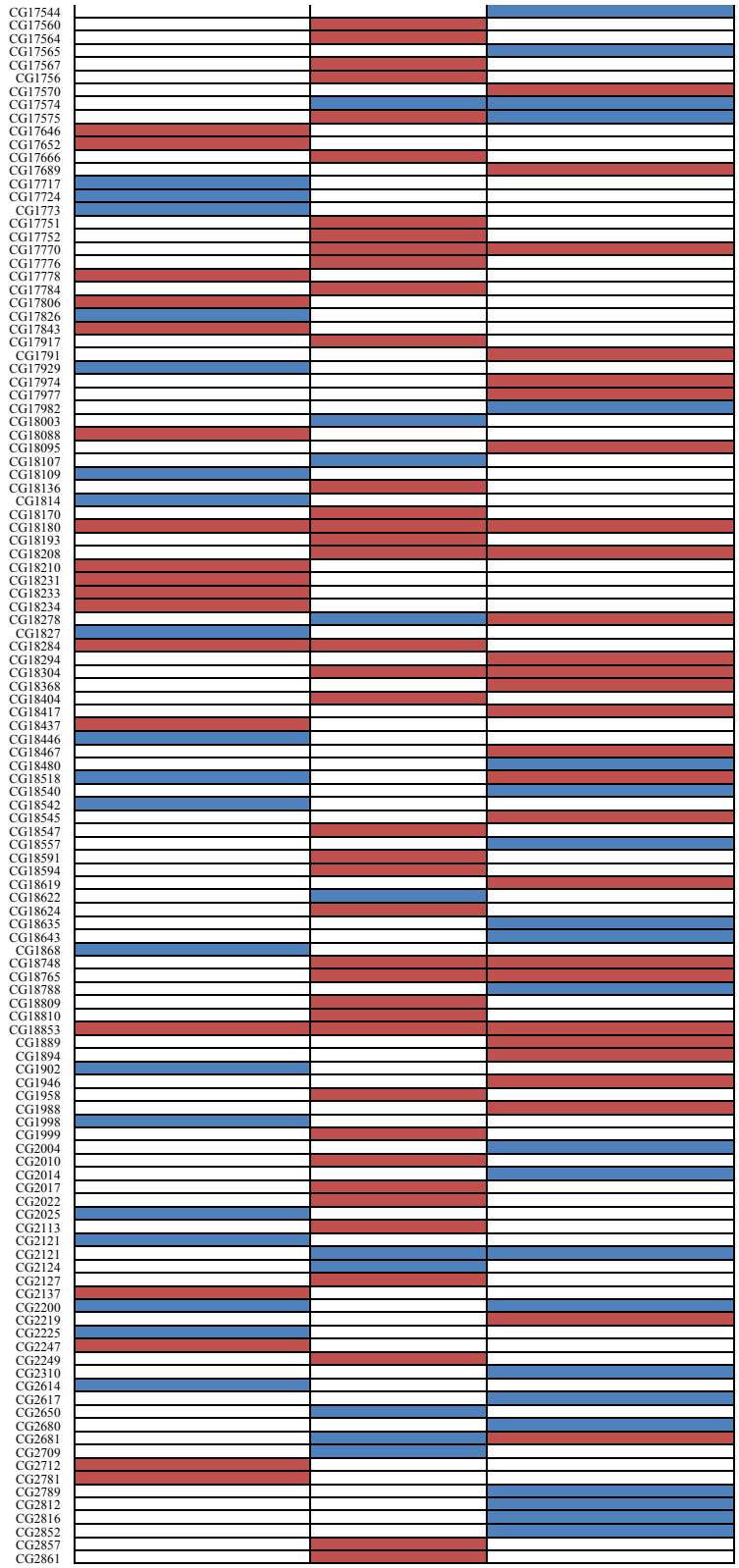
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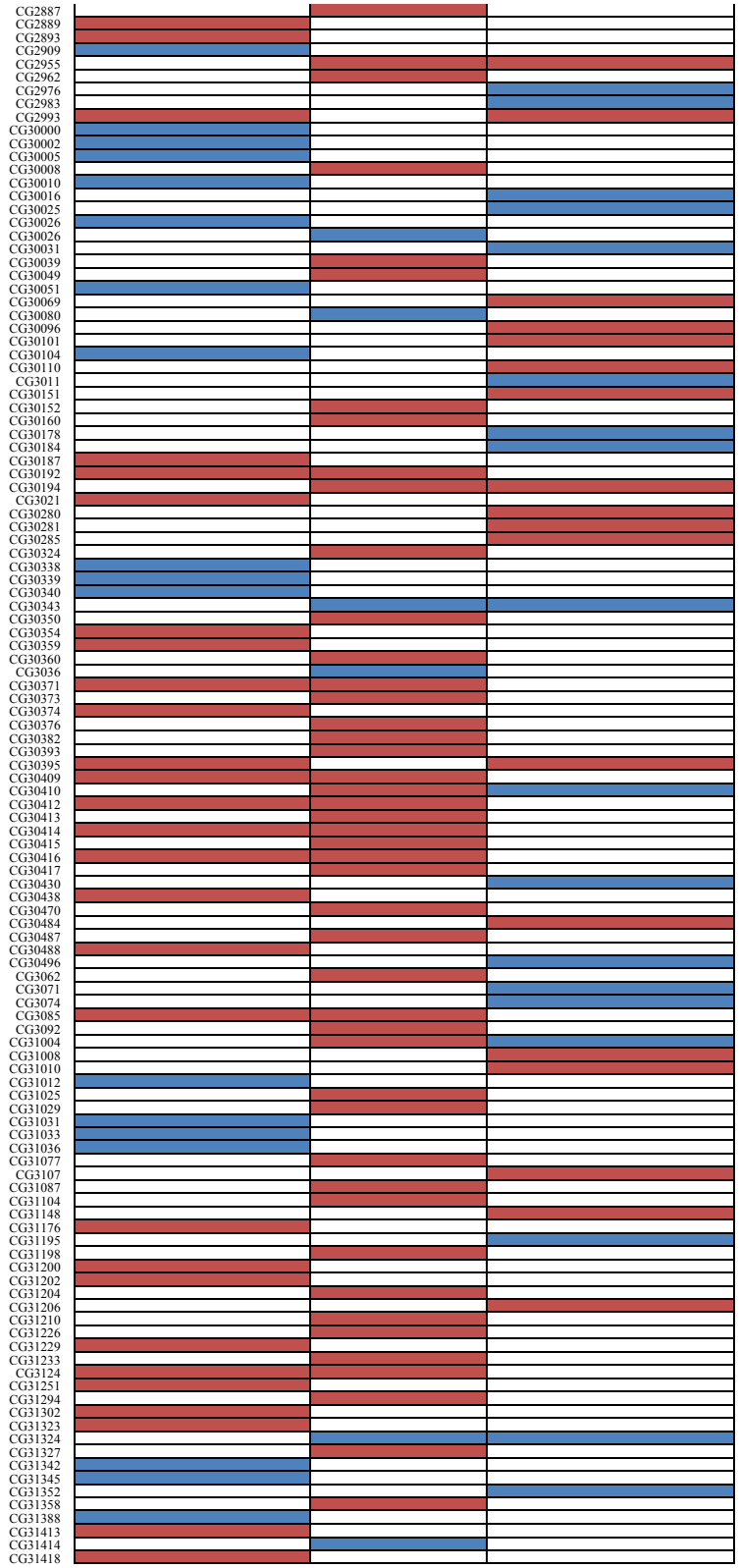
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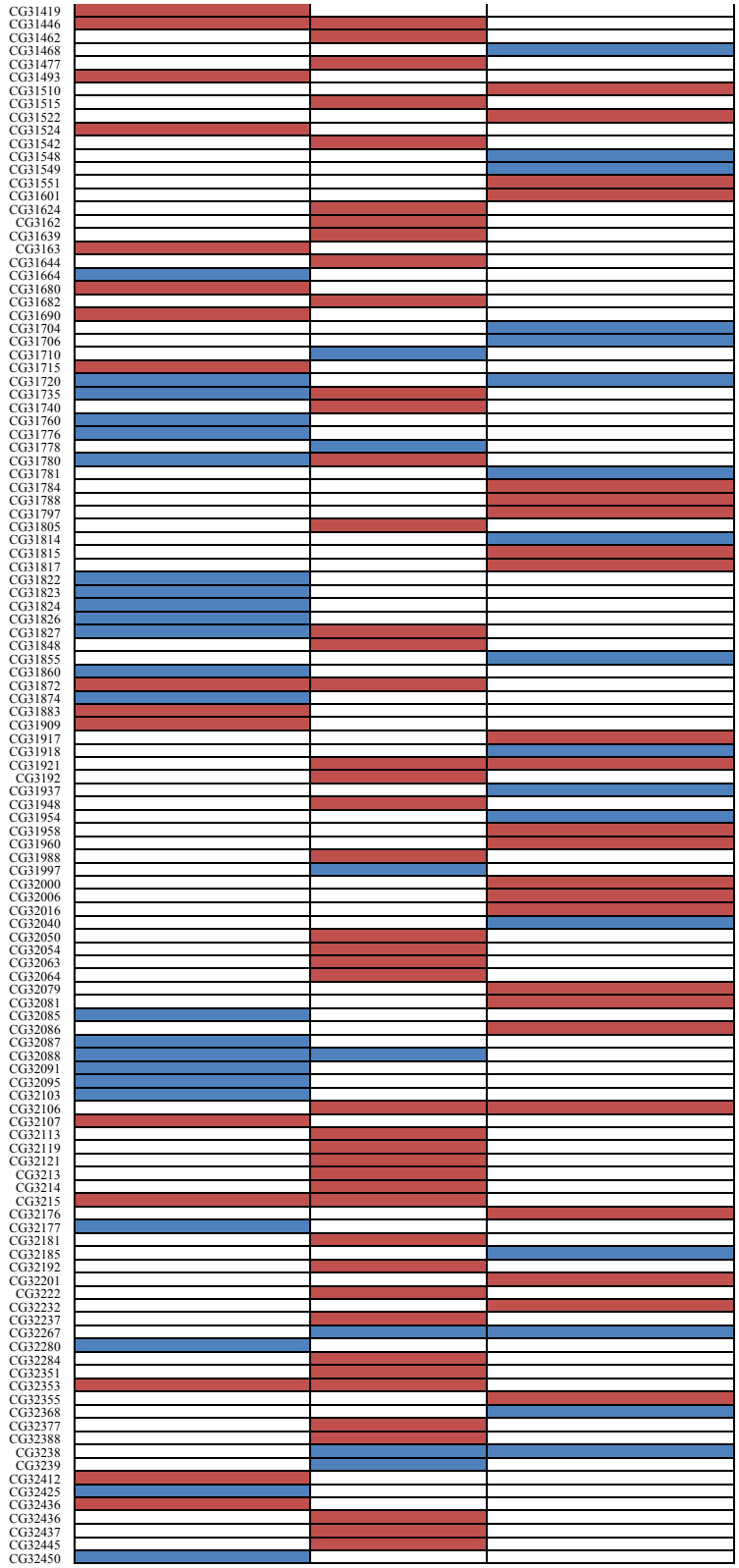
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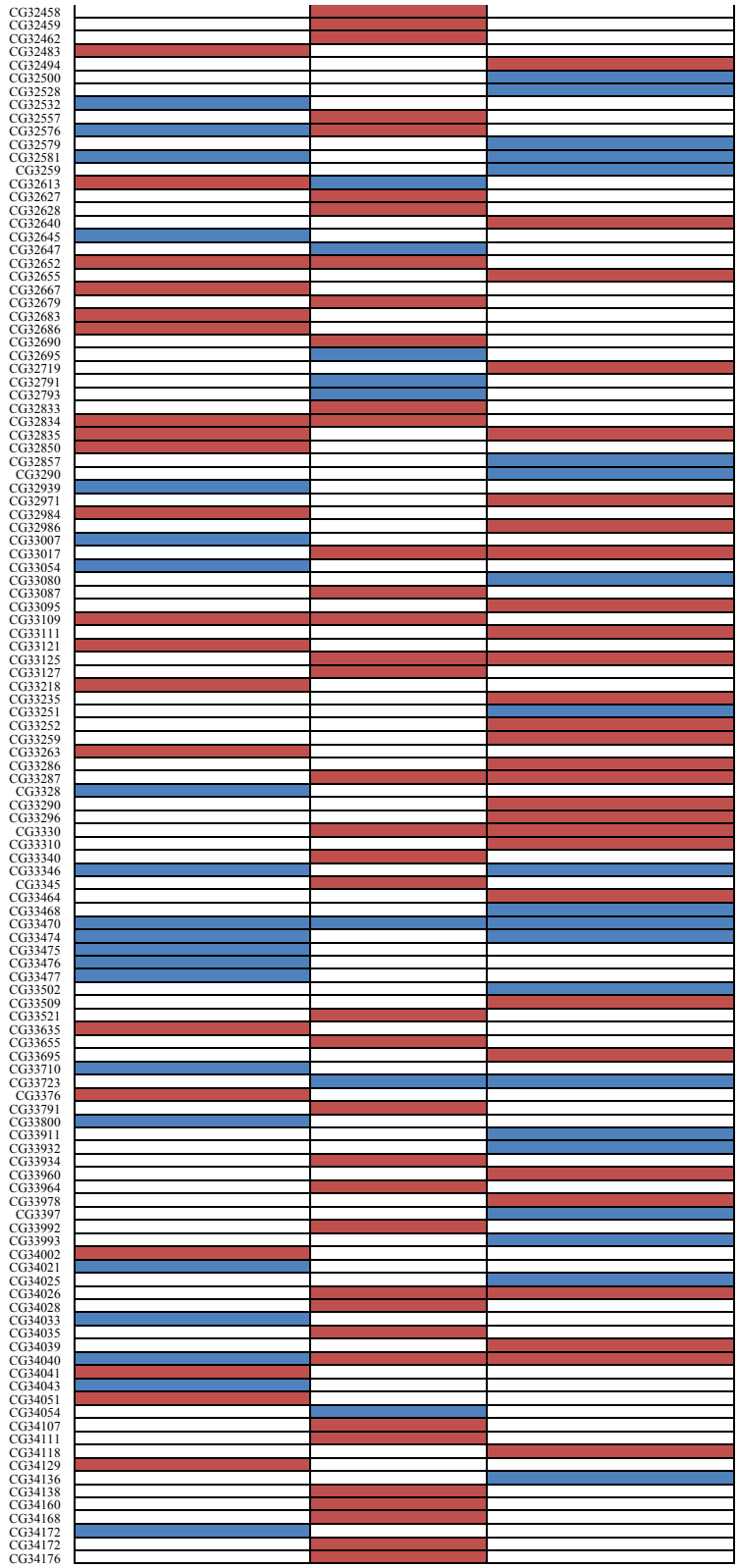
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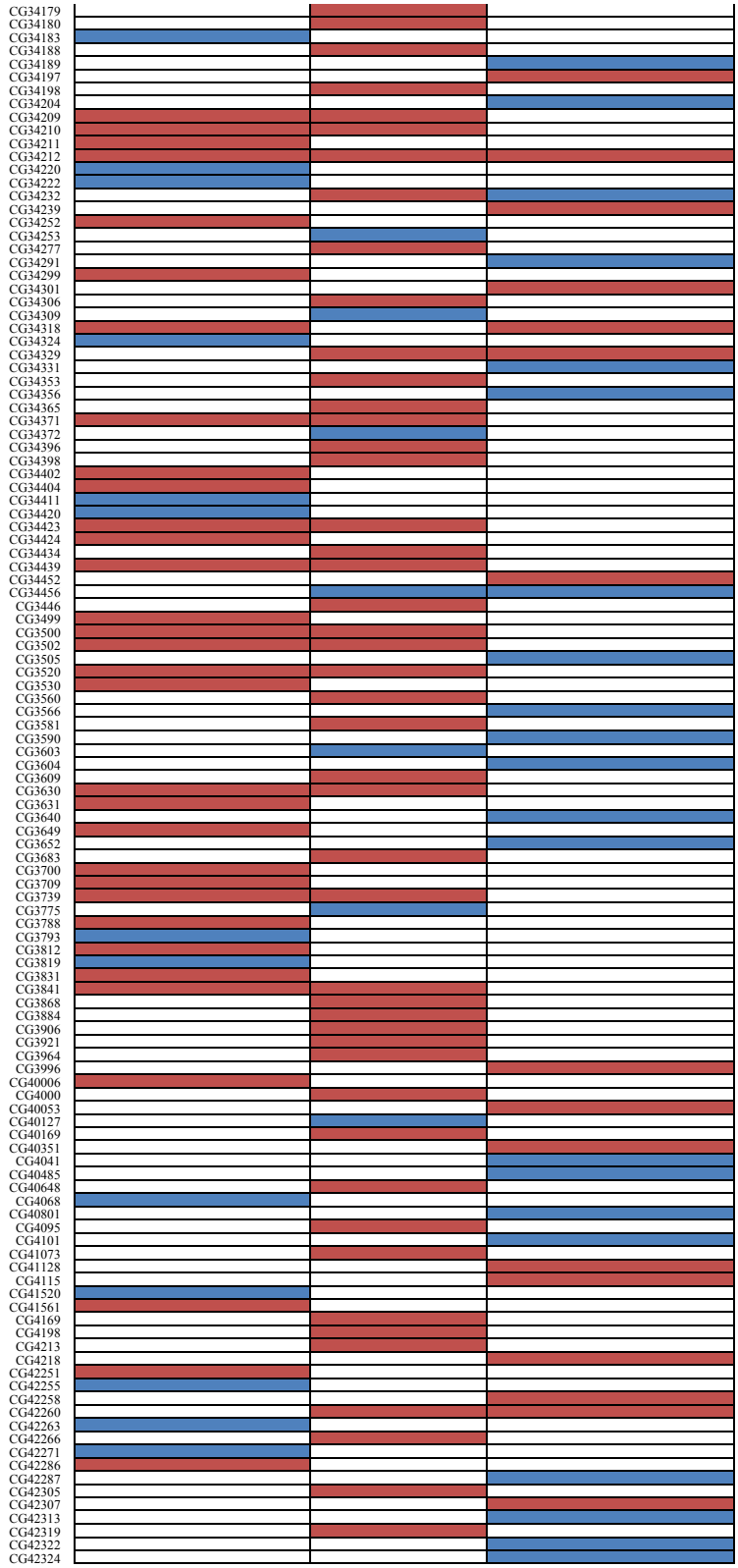
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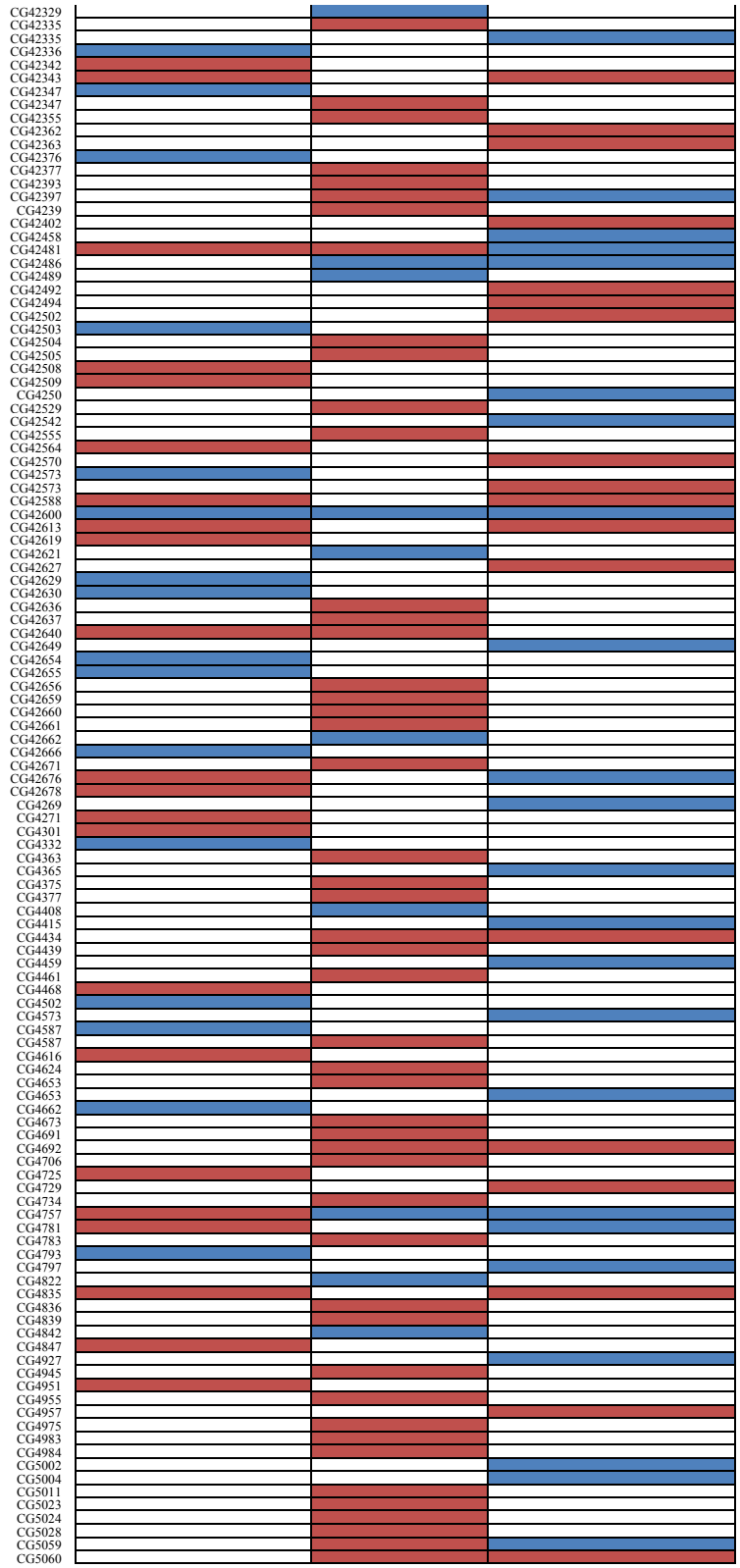
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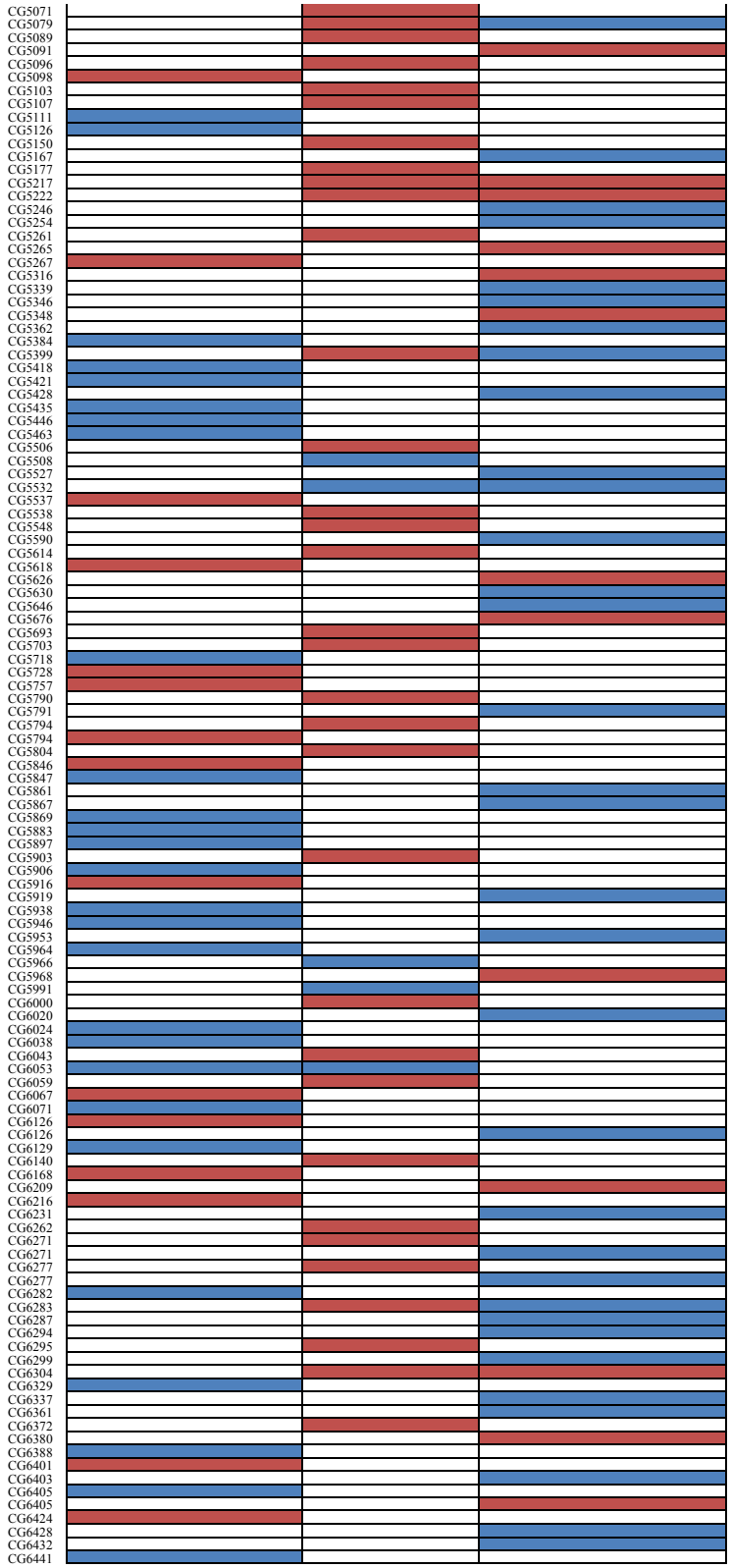
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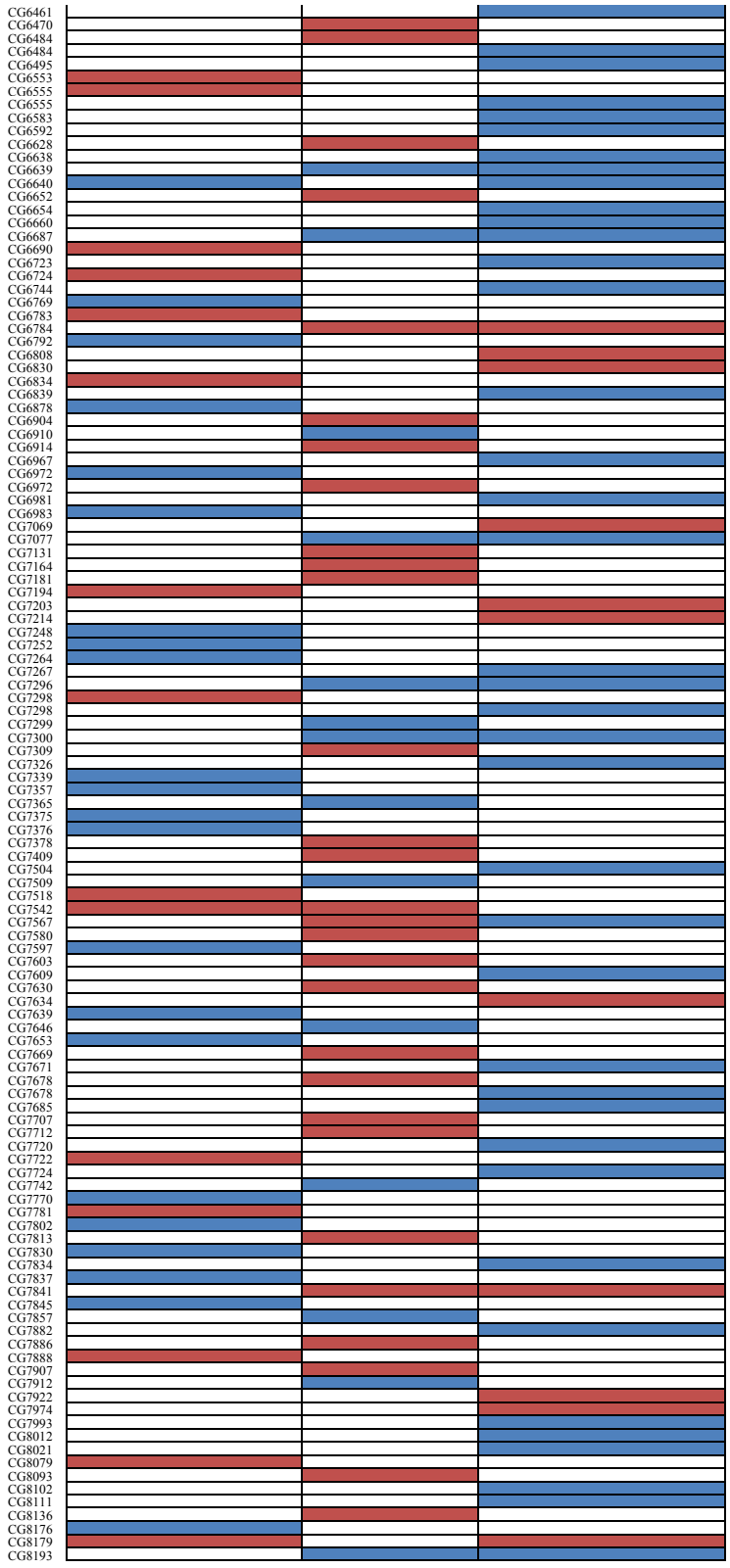
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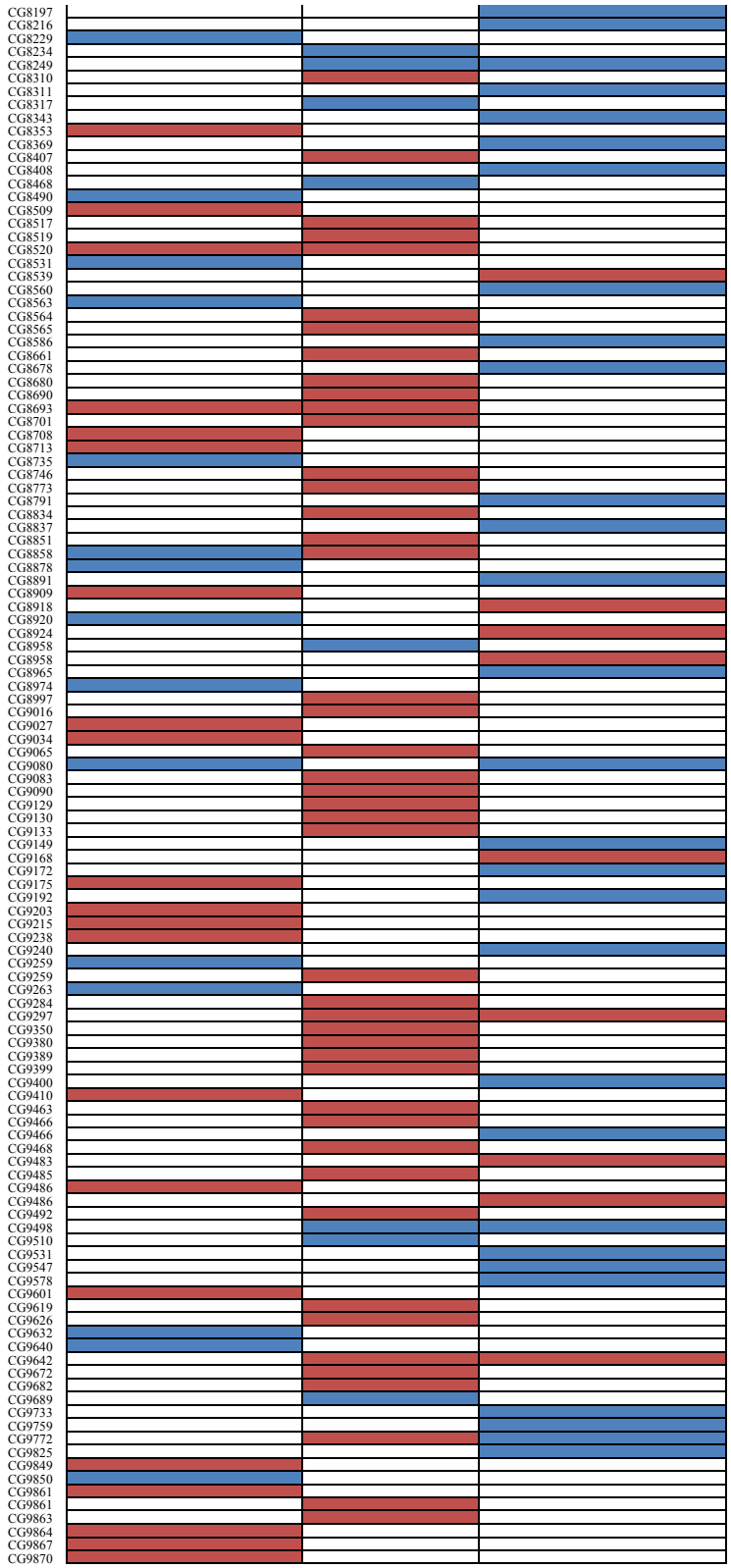
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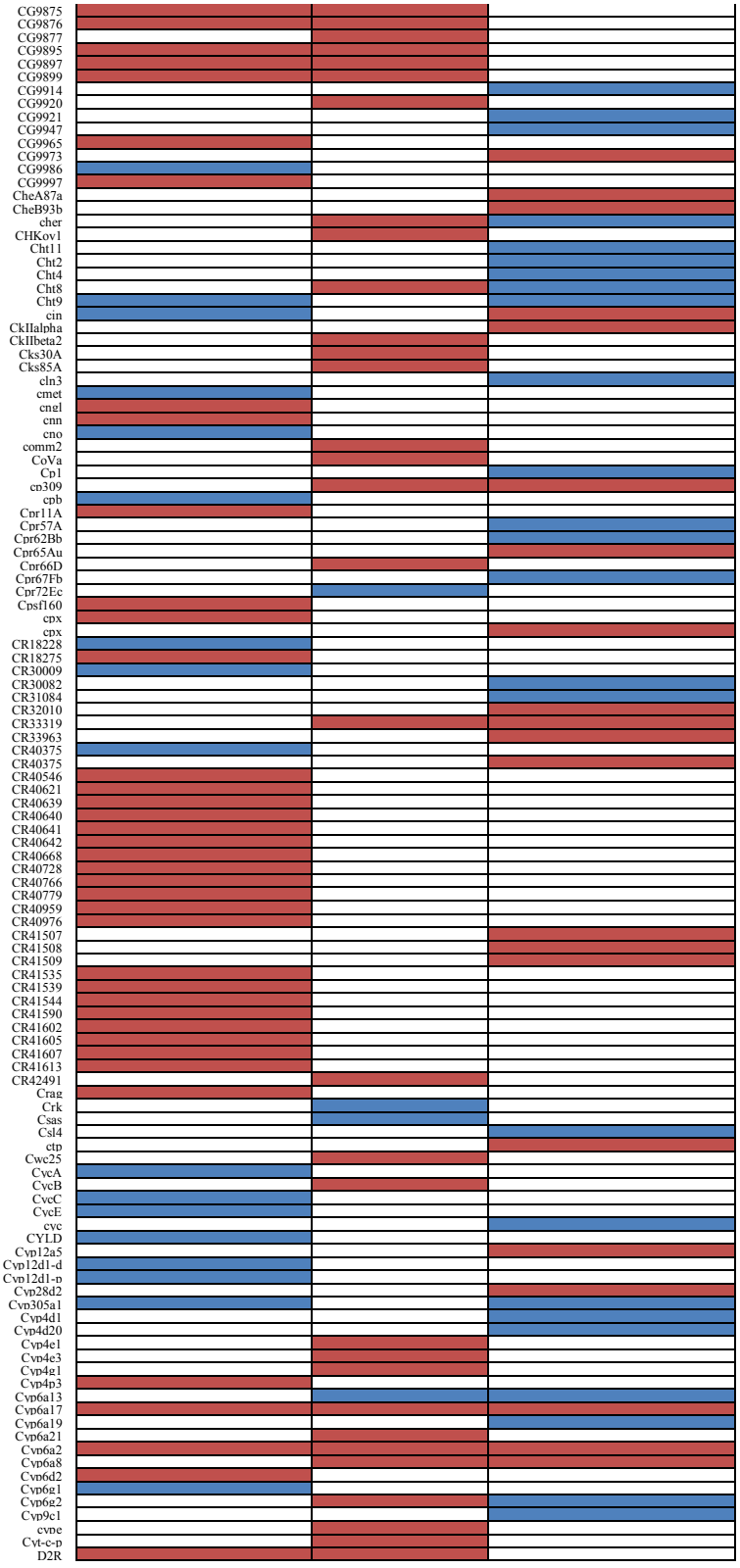
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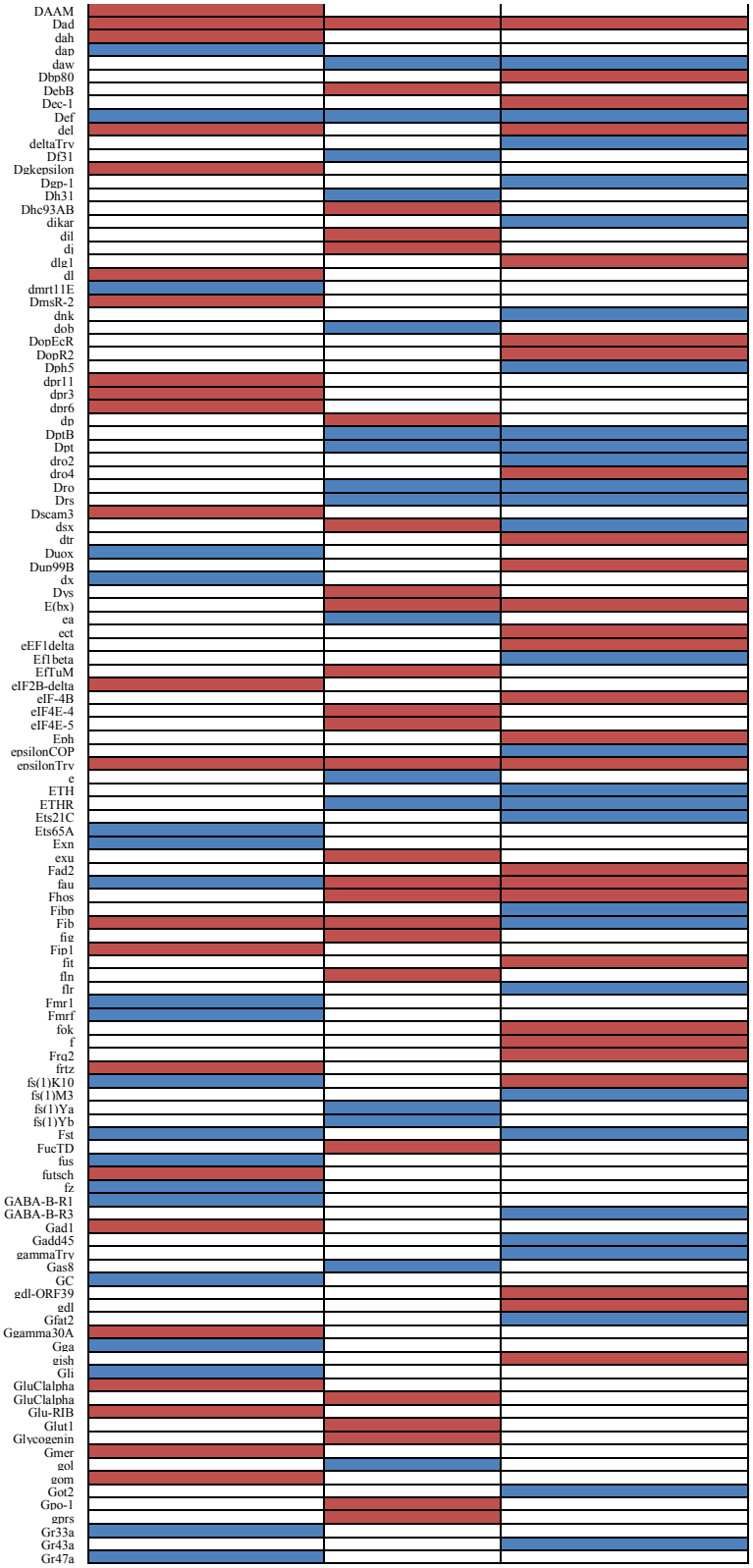
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DopEcR
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dnr6
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DptB
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Dun99B
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eIF-4B
eIF4E-4
eIF4E-5
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epsilonCOP
epsilonTrv
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ETHR
Ets21C
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Frq2
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fs(1)M3
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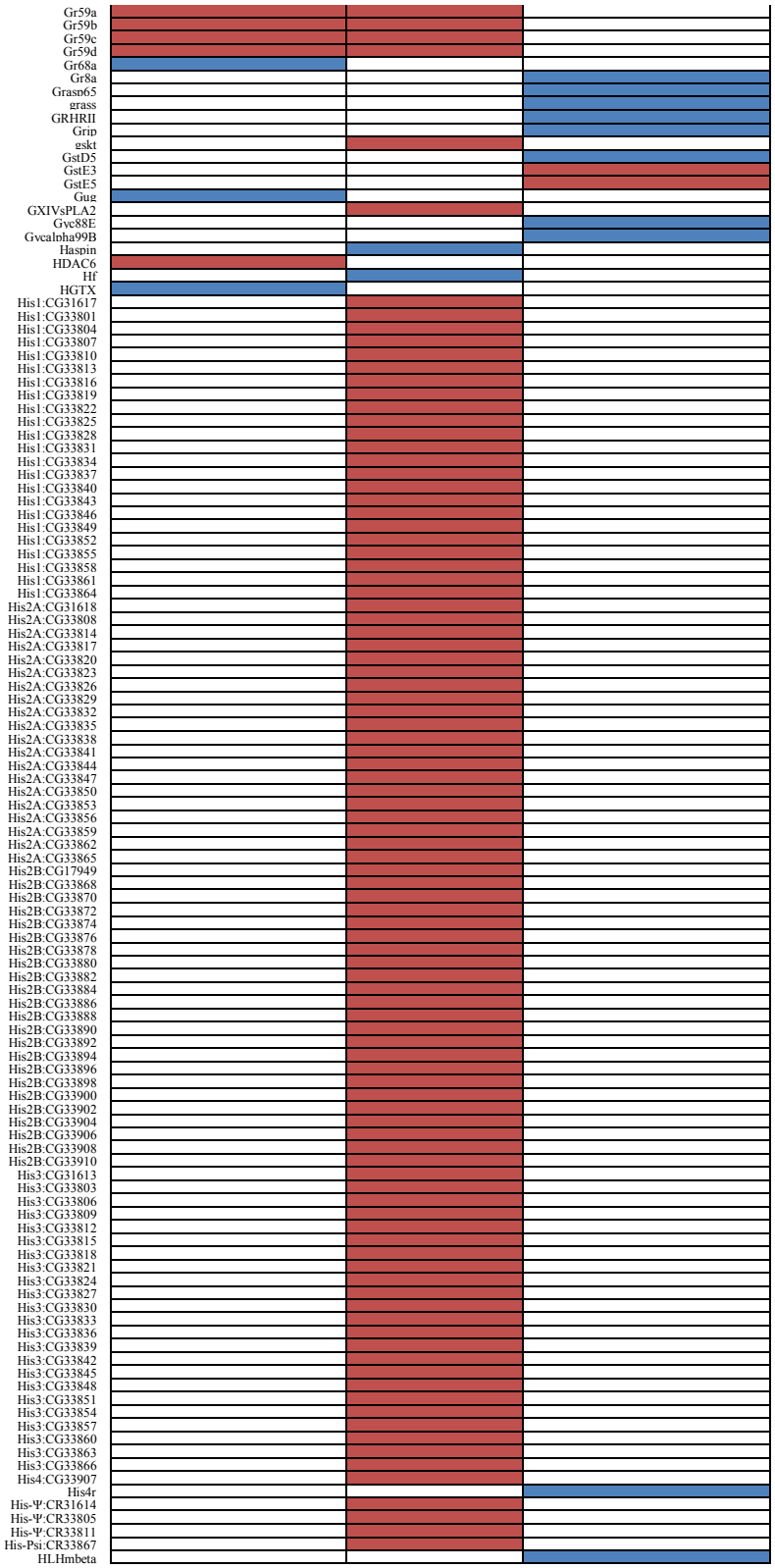
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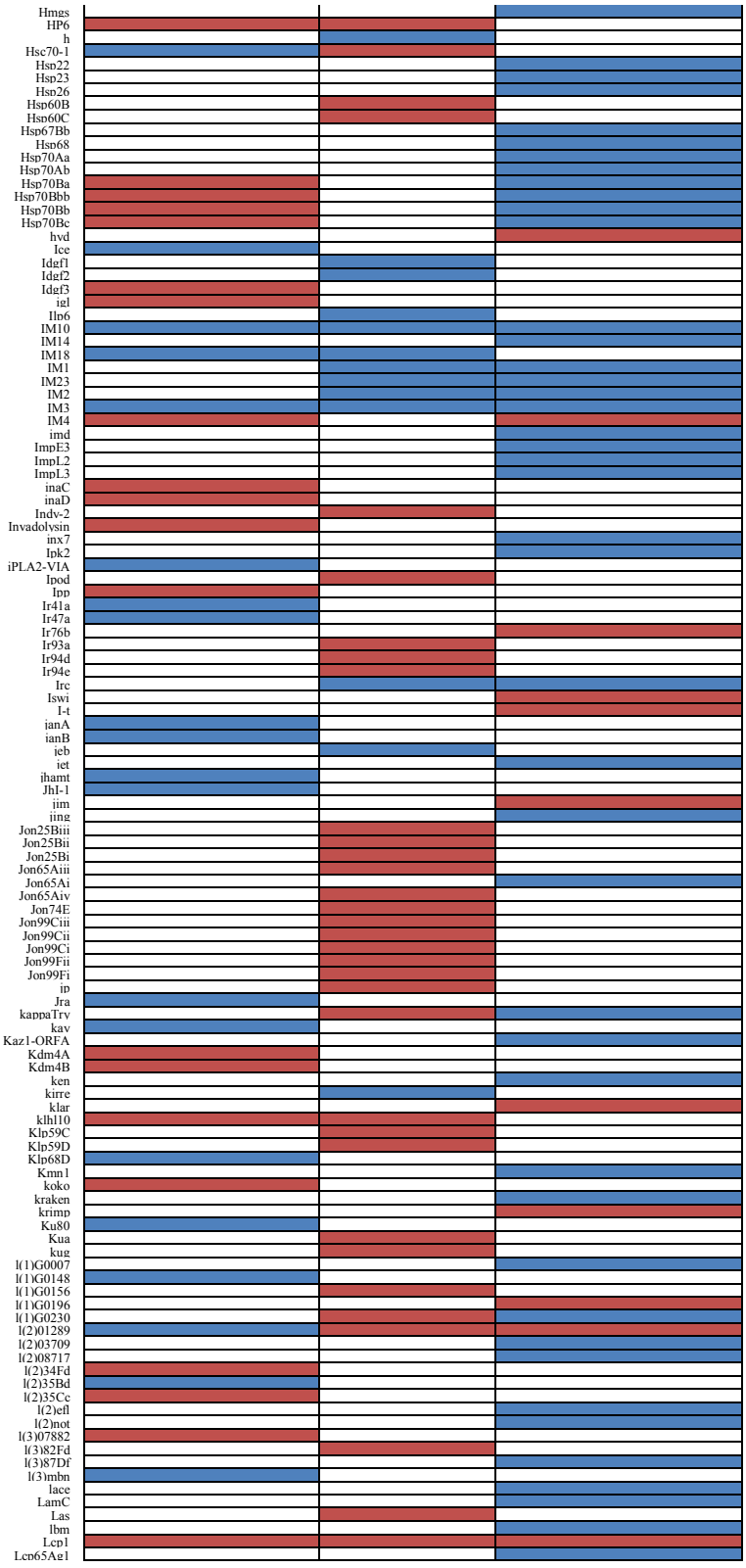
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Iln6
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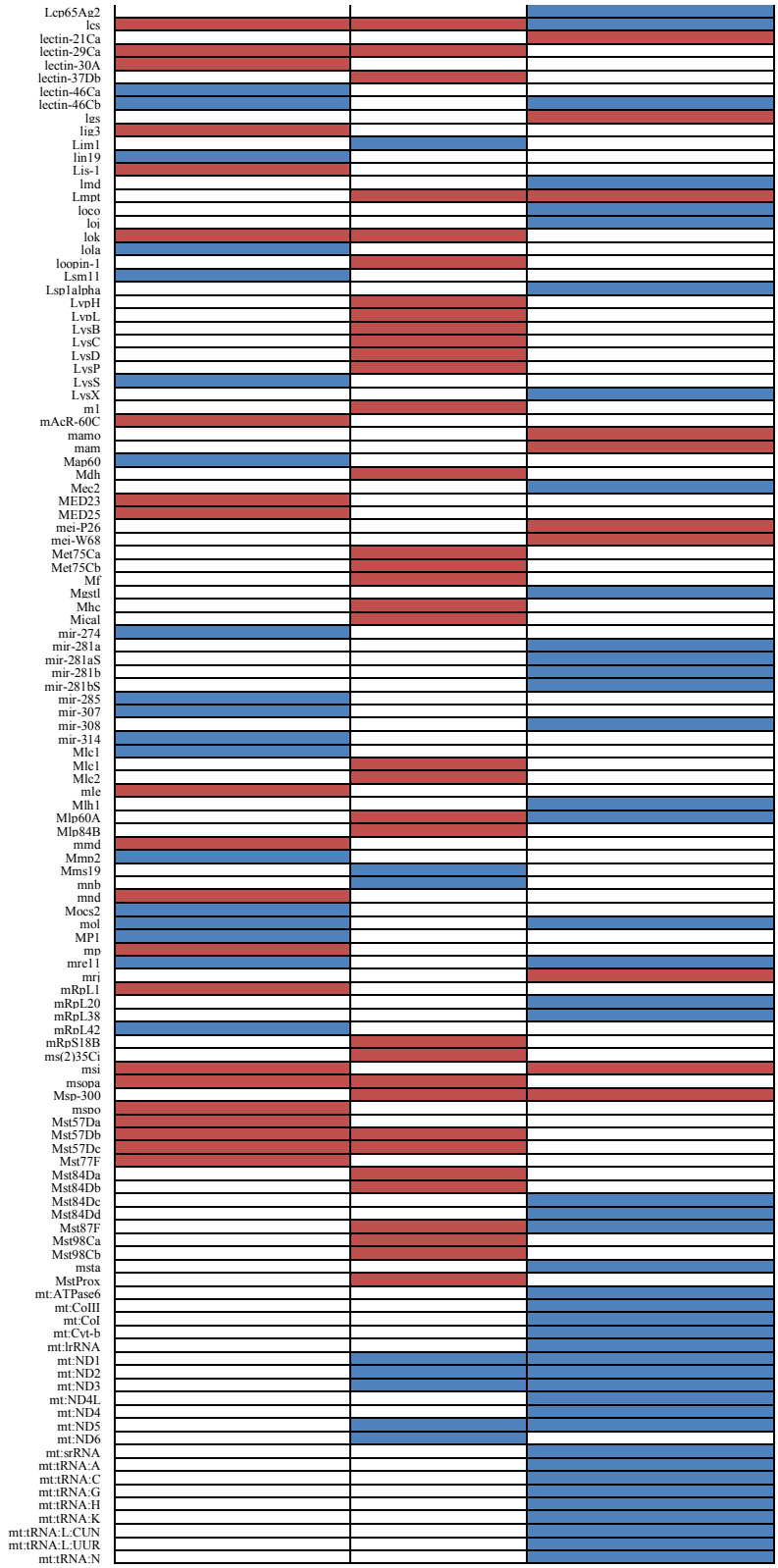
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LysS
LysX
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Mec2
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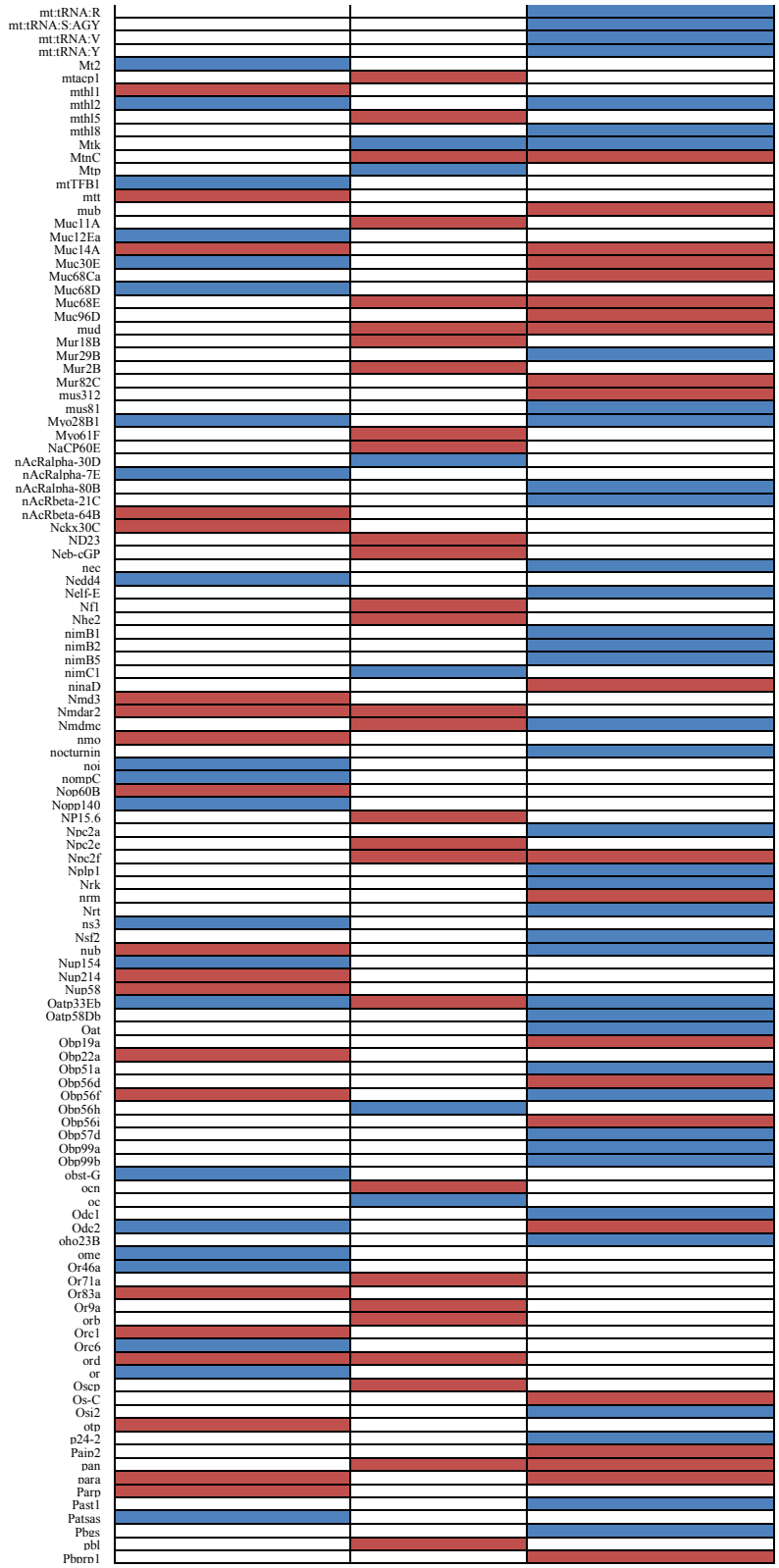
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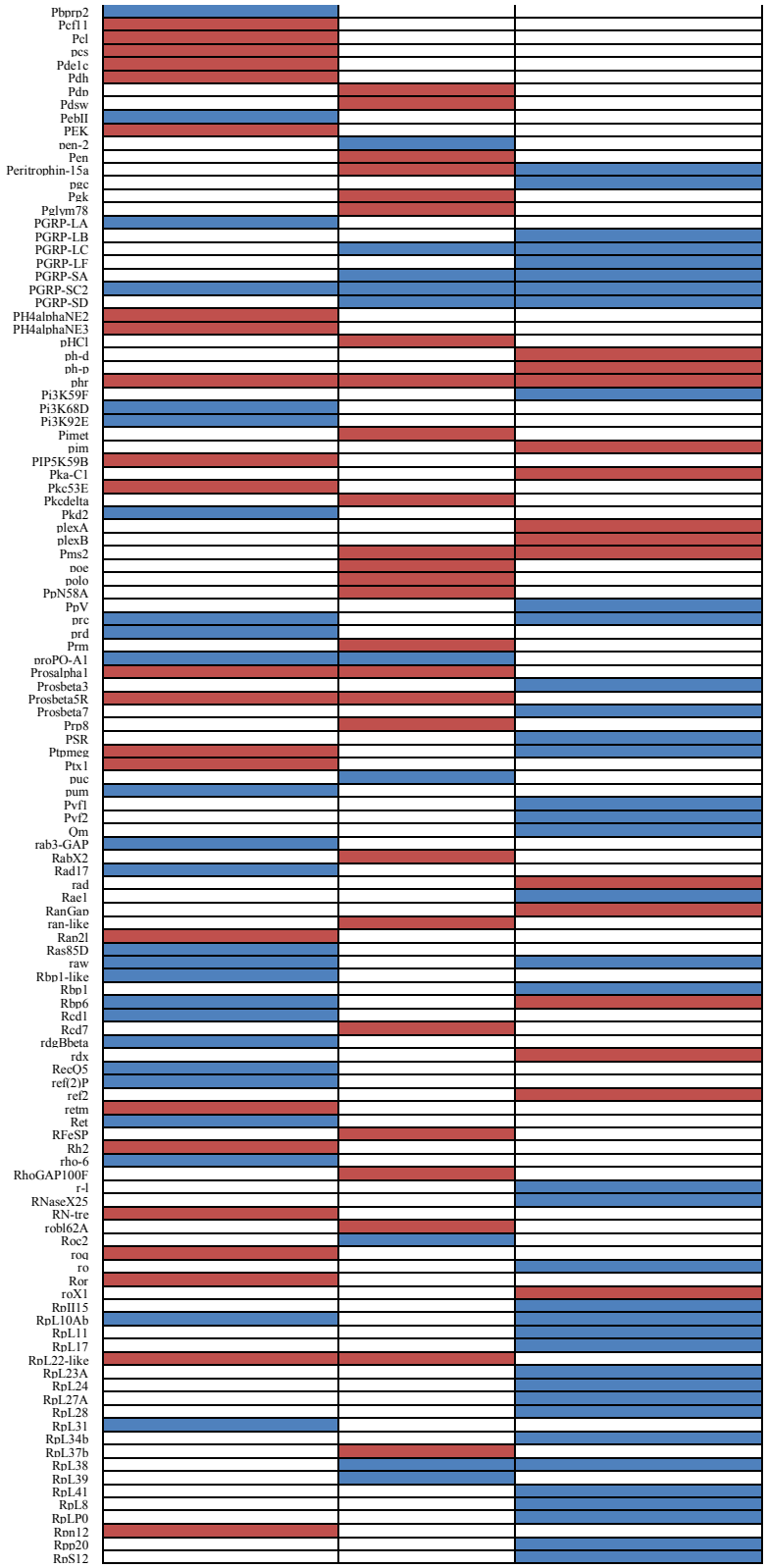
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PH4alphaNE3
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ph-d
ph-p
phr
Pi3K59F
Pi3K68D
Pi3K92E
Pimet
pim
PIP5K59B
Pka-C1
Pkc53E
Pkcdelta
Pkd2
plexA
plexB
Pms2
poe
polo
PpN58A
PpV
prc
prd
Prm
proPO-A1
Prosalpha1
Prosbeta3
Prosbeta5R
Prosbeta7
Prp8
PSR
Ptpmeg
Ptx1
puc
pum
Pvf1
Pvf2
Qm
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RabX2
Rad17
rad
Rae1
RanGap
ran-like
Ran2l
Ras85D
raw
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Rbp1
Rbp6
Rcd1
Rcd7
rdgBbeta
rdx
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retm
Ret
RFeSP
Rh2
rho-6
RhoGAP100F
r-l
RNaseX25
RN-tre
robl62A
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roq
ro
Ror
roX1
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RpL11
RpL17
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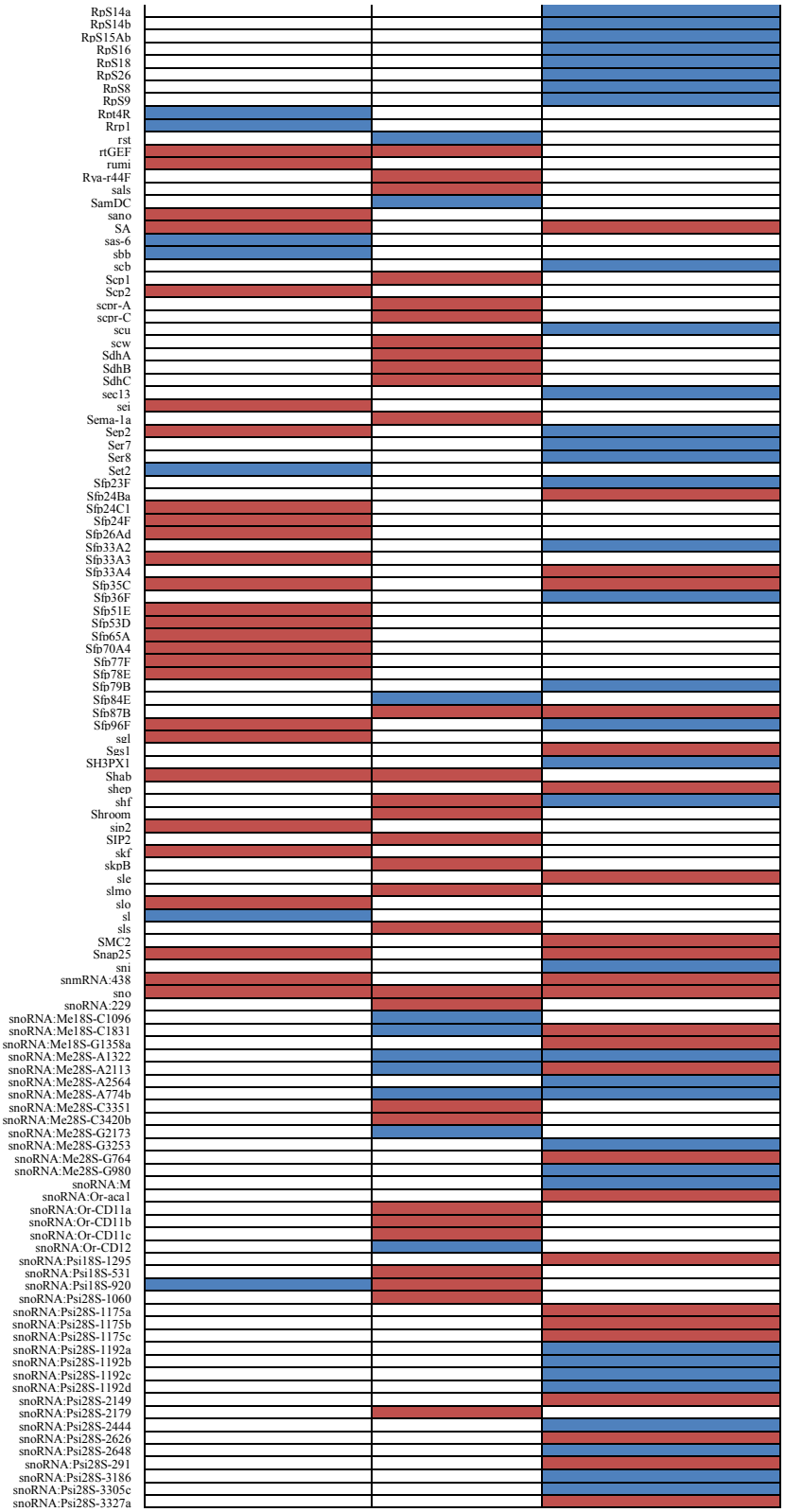
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Scp2
scpr-A
scpr-C
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Sep2
Ser7
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skpB
sle
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slo
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sls
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snoRNA:Me28S-A2113
snoRNA:Me28S-A2564
snoRNA:Me28S-A774b
snoRNA:Me28S-C3351
snoRNA:Me28S-C3420b
snoRNA:Me28S-G2173
snoRNA:Me28S-G3253
snoRNA:Me28S-G764
snoRNA:Me28S-G980
snoRNA:M
snoRNA:Or-aca1
snoRNA:Or-CD11a
snoRNA:Or-CD11b
snoRNA:Or-CD11c
snoRNA:Or-CD12
snoRNA:Psi18S-1295
snoRNA:Psi18S-531
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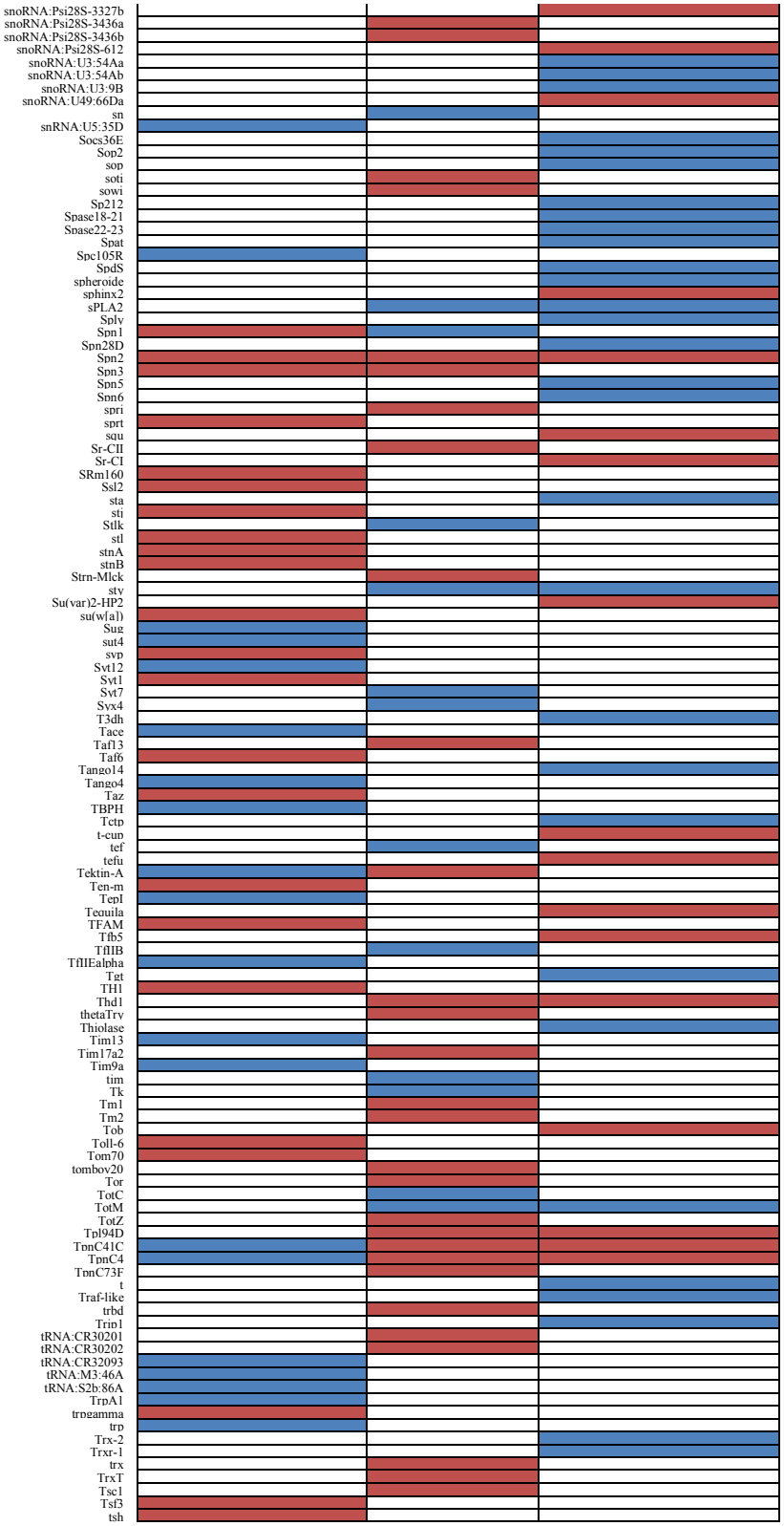
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sPLA2
Splv
Spn1
Spn28D
Spn2
Spn3
Spn5
Spn6
spri
sprt
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Sr-CI
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Svt1
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Svx4
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Tace
Taf13
Taf6
Tango14
Tango4
Taz
TBPH
Tctp
t-cun
tef
tefu
Tektin-A
Ten-m
TepI
Tequila
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TfIIB
TfIIEalpha
Tgt
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Thd1
thetaTrv
Thiolase
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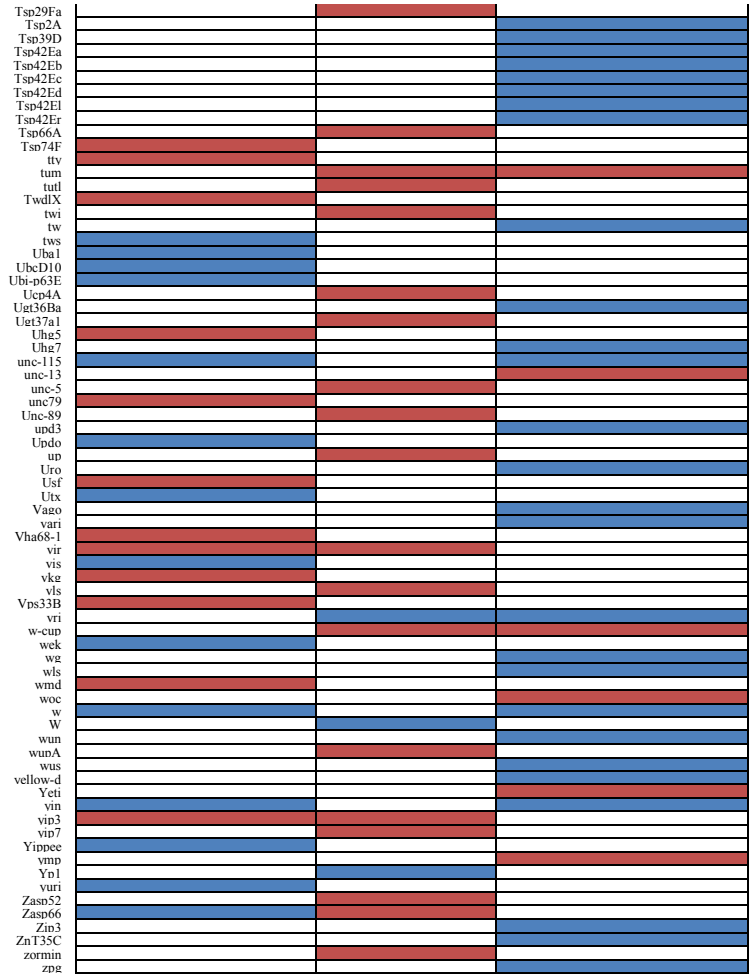
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Ubi-p63E
Ucp4A
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Ugt37a1
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Updo
up
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Usf
Utx
Vago
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Vha68-1
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vis
vkg
vls
Vps33B
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w-cup
wek
wg
wls
wmd
woc
w
W
wun
wunA
wus
yellow-d
Yeti
yin
yip3
yip7
Yippee
ymp
Yp1
vuri
Zasp52
Zasp66
Zip3
ZnT35C
zormin
zpg

Figure 3-24.

Table 3-1. Male accessory gland specific genes expressed in male corpora allata.

Gene	Gene Symbol	Gene Name	DB identifier
CG42543	mp	multiplexin	FBgn0260660
CG9151	acj6	abnormal chemosensory jump 6	FBgn0000028
CG5201	Dad	Daughters against dpp	FBgn0020493
CG11680	mle	maleless	FBgn0002774
CG9907	para	paralytic	FBgn0260993
CG10693	slo	slowpoke	FBgn0003429
CG3578	bi	bifid	FBgn0000179
CG11491	br	broad	FBgn0000210
CG9876	CG9876		FBgn0034821
CG42741	CG42741		FBgn0261705
CG6667	dl	dorsal	FBgn0260632
CG3668	fd59A	forkhead domain 59A	FBgn0004896
CG10593	Acer	Angiotensin-converting enzyme-related	FBgn0016122
CG8982	Acp26Aa	Accessory gland-specific peptide 26Aa	FBgn0002855
CG13095	Bace	beta-site APP-cleaving enzyme	FBgn0032049
CG12736	CG12736		FBgn0033184
CG13802	CG13802		FBgn0035330
CG14061	CG14061		FBgn0039598
CG14701	CG14701		FBgn0037883
CG16798	CG16798		FBgn0032856
CG2681	CG2681		FBgn0024997
CG30371	CG30371		FBgn0050371
CR30374	CR30374		FBgn0050374
CG30412	CG30412		FBgn0050412
CG31176	CG31176		FBgn0051176
CG32436	CG32436		FBgn0052436
CG32483	CG32483		FBgn0052483
CG32613	CG32613		FBgn0052613
CG3502	CG3502		FBgn0046253
CG4468	CG4468		FBgn0038749
CG4835	CG4835		FBgn0035607
CG4847	CG4847		FBgn0034229
CG9034	CG9034		FBgn0040931
CG3504	inaD	inactivation no afterpotential D	FBgn0001263
CG3953	Invadolysin	Invadolysin	FBgn0086359
CG17227	lig3	DNA ligase III	FBgn0038035
CG10895	lok	loki	FBgn0019686

CG4356	mAcR-60C	muscarinic Acetylcholine Receptor 60C	FBgn0000037
CG3695	MED23	Mediator complex subunit 23	FBgn0034795
CG12254	MED25	Mediator complex subunit 25	FBgn0038760
CG11348	nAcRbeta-64B	nicotinic Acetylcholine Receptor beta 64B	FBgn0000038
CG40411	Parp	Poly-(ADP-ribose) polymerase	FBgn0010247
CG12758	sano	serrano	FBgn0034408
CG3423	SA	Stromalin	FBgn0020616
CG14904	Scp2	Sarcoplasmic calcium-binding protein 2	FBgn0020907
CG3182	sei	seizure	FBgn0003353
CG4173	Sep-2	Septin-2	FBgn0014029
CG42466	Sfp24C1	Seminal fluid protein 24C1	FBgn0259956
CG42468	Sfp24F	Seminal fluid protein 24F	FBgn0259958
CG42603	Sfp26Ad	Seminal fluid protein 26Ad	FBgn0261055
CG42474	Sfp33A3	Seminal fluid protein 33A3	FBgn0259964
CG42475	Sfp35C	Seminal fluid protein 35C	FBgn0259965
CG42476	Sfp51E	Seminal fluid protein 51E	FBgn0259966
CG42477	Sfp53D	Seminal fluid protein 53D	FBgn0259967
CG40452	Snap25	Synapse protein 25	FBgn0011288

Table 3-1.

Table 3-2. Differentially expressed genes in whole flies after ETHR-RNAi which regulate reproductive behavior. Differentially expressed genes involved in reproduction determined by a less stringent multiple hypothesis test at p- value <0.05.

Up-regulated Genes	Down-regulated Genes
Acp98AB	Acp29AB
aPKC	BG642312
capt	brm
CG17575	cac
CG2852	ci
Cg31704	cic
Cg43319	ctp
CG6555	dec-1
cher	del
clos	dlg1
dsx	Dup99B
fs(1)M3	dup99B
jing	Fad2
ken	fs(1)K10
lectin-46Cb	gdl
loco	gdl-ORF39
loj	gish
mt:IrRNA	hyd
mt:srRNA	krimp
Obp51a	mamo
Obp56f	mei-P26
Past1	mei-W68
pgc	msi
Pvf1	Msp-300
Sfp36F	para
Sfp79B	ph-p
Sfp93F	Pka-C1
Sfp96F	sno
Sop2	Spn2
sty	squ
w	
wg	
wun	
zpg	

Table 3-2.

Table 3-3. Male-male interaction genes from (Ellis and Carney 2011) differentially expressed genes in three samples tested.

Gene	Ellis and Carney Male-male Interaction	CA	Head	Whole fly
Tequila	Up	-	-	Down
Las	Up	-	Down	-
Np15.6	Up	-	Down	-
comm2	Up	-	Down	-
Mtp	Up	-	Up	-
Cyp6a21	Up	-	Down	-
CG13369	Up	Up	-	-
CG14823	Up	-	-	Down
CG15199	Up	-	Up	-
CG15201	Up	-	-	Up
GstE5	Up	-	-	Down
SmE	Up	-	Down	-
CG30382	Up	-	Down	-
dlg1	Down	-	-	Down
Mmp2	Down	Up	-	-
sls	Down	-	Down	-
PIP5K59B	Down	Down	-	-
Bruce	Down	-	Down	-
Pkc53E	Down	Down	-	-
ctp	Down	-	-	Down
CadN	Down	-	Down	Down
pcs	Down	Down	-	-
trp	Down	Up	-	-
nmo	Down	Down	-	-
RN-tre	Down	Down	-	-
CG8878	Down	Up	-	-
Ras85D	Down	Up	-	-
fz2	Down	Up	-	-
CG10631	Down	Down	-	-
CG11760	Down	Up	-	-
lola	Down	Up	-	-
CG12605	Down	Down	-	-
CG14411	Down	Up	-	-
plexB	Down	-	-	Down
sprt	Down	Down	-	-
CG31760	Down	Up	-	-
Mical	Down	-	Down	-

Unc-89	Down	-	Down	-
NaCP60E	Down	-	Down	-
Msp-300	Down	-	Down	Down

Table 3-3.

Table 3-4. Differentially expressed courtship-song transcripts with their respective fold changes and p-values in three samples. (-) indicates no change. Italicized numbers: p-values.

Transcript	CA	Head	Whole Flies
cac-RA	-	-	-2.15 *(0.00491)*
cac-RB	-	-	-2.15 *(0.00491)*
cac-RC	-	-	-2.16 *(0.00471)*
cac-RD	-	-	-2.16 *(0.00471)*
cac-RE	-	-	-2.15 *(0.00491)*
cac-RF	-	-	-2.16 *(0.00471)*
cac-RG	-	-	-2.16 *(0.00471)*
cac-RH	-	-	-2.15 *(0.00491)*
para-RA	-2.49 *(0.05)*	-	-
para-RB	-3.06 *(0.04)*	-	-1.53 *(0.09)*
para-RC	-2.74 *(0.03)*	-	-
para-RD	-3.78 *(0.01)*	-	-
FoxP-RB	-	-	-2.76 *(0.002)*

Table 3-4.

Table 3-5. *Doublesex* exon specific normalized number of reads from head library.

***dsx* Exon Number**	**Male/Female**	**Start Location**	**End Location**	**Normalized Read Number**
1	Both	3792185	3793130	20
2	Both	3785474	3786844	139
3	Both	3761490	3761627	26
4	Male only	3755893	3756409	51
5	Male only	3750045	3751082	139
6	Female only	3760200	3761375	111
7	Female only	3787952	3788508	21

Table 3-5.

Table 3-6. Primer sequences with their resective melting temperatures (Tm).

Primer	Sequence	Tm
ETHR-A Frwd	CGATTACTGCTGGAATTGGTGACA	60C
ETHR-A Rev.	TTGAGGAGTTGGTATTCGTGTTCG	60C
ETHR-B Frwd	CCTACAAGCTGCTCCGTCCCA	60C
ETHR-B Rev.	TGCTTGCAGTGCTTCCTCAT	60C
fru Frwd.	CCGCATGCTTGATCTTACAGTG	60C
fru Rev.	CGATGCGTTAGTTGCAACAAGA	60C
dsx Frwd	GCTTAATGCTTCGGTGAAATCG	60C
dsx Rev.	GTTGATTGAAGATAGTCCAAGTCGC	60C
Gr68a Frwd	TCCTATATCCAAGCCCTCGCA	60C
GR68a Rev.	CTGTTGATCTCCTCGGTATCACCT	60C
Actin5C Frwd	CATCCACGAGACCACCTACA	60C
Actin5C Rev.	TTGGAGATCCACATCTGCTG	60C
dsx exon-3 Frwd	GGGCCAAGACGTTTTCCTAGAC	60C
dsx exon-4 Rev.	GTAGATCTGGGCTACAGTGCGA	60C

Table 3-6.

CHAPTER FOUR

CONCLUDING REMARKS

CONCLUDING REMARKS

Peptides play critical roles in behavioral regulation and peptide signaling has been conserved evolutionarily over a wide range of organisms. Organisms use peptide signaling for regulation of behaviors such as communication and mating. Higher organisms such as insects use peptides for multiple purposes; including regulation of critical behaviors (Vezenkov et al. 2009). Behaviors are a complex sequence of motor movements that result from responses to various sensory, mechanical and chemical cues (Siegel et al. 1979; Levine et al. 2002; Carney 2007; Ellis et al. 2009; Ellis et al. 2011). *Drosophila,* due to availability of its genome sequence and molecular-genetic tools, has been widely used as a model organism to study neuronal and molecular mechanisms of peptidergic behavioral regulation (Carvalho et al. 2006; Kim et al. 2006).

Courtship in *Drosophila* is an innate behavior, where the male performs a sequence of stereotypic behavioral subunits. The male orients towards the female, taps her on the abdomen, sings the courtship song by vibrating one wing, licks the female abdomen and finally attempts to copulate. This sequence of events occurs in response to various auditory, visual, olfactory and gustatory cues (Greenspan et al. 2000; Billeter, Goodwin et al. 2002; Dahanukar et al. 2011). Genes that speficy sex determination, *fru* and *dsx* are expressed in sensory neurons and are known to affect courtship song and behavior (Billeter et al. 2002; Rideout et al. 2007). Other genes regulating male courtship behavior encode olfactory and gustatory receptors such as *Or67d, Or65a, Gr33a, Gr32a* and *Gr68a* (Bray et al. 2003; Kurtovic et al. 2007; Miyamoto et al. 2008; Moon et al.

2009). Expression of *Gr68a* is regulated by *dsx* (Bray et al. 2003). Molting hormones including juvenile hormone and ecdysone also are known to regulate of male courtship behavior (Ganter et al. 2007; Liu et al. 2008; Dalton et al. 2009; Ishimoto et al. 2009; Gante et al. 2011).

Ecdysis, the process by which insects shed their exocuticle, is another innate behavior regulated by various hormones (Zitnan et al. 2007). The peptide ecdysis triggering hormone (ETH) acts as a command chemical to regulate ecdysis behavior (Park et al. 2002; Kim et al. 2006). ETH is produced by Inka cells present in the epitracheal gland. Interestingly, Inka cells persist in the adult stage, where ecdysis no longer occurs. In order to check how long in the adult stage expression of ETH expression persists, *ETH-Gal-4* driven expression of the fluorophore tdTomato was checked at different adult stages in both the sexes. Results of these experiments show that expression of ETH in Inka cells occurs in both sexes at day 1, 5 and 15 after eclosion, demonstrating a likely role for ETH signaling in adult *Drosophila*. In a search for novel functions of ETH signaling, I focused on behaviors of adult flies after silencing of ETH receptors and altered gene expression patterns resulting from ETH receptor silencing.

In immature stages ETH triggers central peptidergic signaling cascades leading to the ecdysis behavioral sequence (Kim et al. 2006). ETHRs are G-protein coupled receptors that signal through the Gαq pathway to mobilize intracellular calcium stores. Most ETHR neurons are peptidergic, and release peptides which further regulate ecdysis behavior. Recently, ETHRs were found to be expressed in corpora allata of the silkworm

Bombyx. In order to elucidate the role of ETHRs in corpora allata, ETHRs were silenced in corpora allata (CA) using the *Aug21-Gal-4* driver line. *Aug21-Gal-4* is a known CA specific driver for larval stages of *Drosophila*. Silencing of ETHRs in *Drosophila* results in elevated male-male courtship, but does not affect female behaviors. RNAseq data from various *Drosophila* stages shows the absence of ETHR transcripts in females after day 3 (Graveley et al. 2011); this may explain the lack of phenotype in females after ETHR-RNAi.

In order to check the expression pattern of *Aug21-Gal-4* in the adult stage, immunostaining of *Aug21-Gal-4* induced GFP expression was done in the adult *Drosophila*. Surprisingly, GFP expression was found not only in the CA, but also in a subset of central neurons. This posed a challenge to identify these *Aug21-Gal-4* labeled neurons. Due to the lack of authentic CA-specific line, I was compelled to test various Gal-4 lines and to make a CA specific Gal-4 line. An attempt to make a CA specific Gal-4 line was done using the sequence of a gene involved in the juvenile hormone synthesis pathway. Acetoacetyl CoA thiolase (AACT) is an enzyme that regulates the first step in the mevalonate pathway. It regulates the conversion of Acetyl-CoA into Acetoacetyl-CoA (Bellás et al. 2005). AACT transcript expression, being specific in *Bombyx* CA (Kinjoh et al. 2007) was the best candidate for making a CA specific Gal-4 line in *Drosophila*. The AACT gene sequence was taken from www.flybase.org and primers were designed to clone the AACT gene. After confirming the sequence, *AACT* was cloned into a Gal-4 vector and *AACT-Gal-4* fly lines were generated by sending out the

plasmid for egg injections. *AACT-gal-4* expression was checked by immunostaining GFP expression driven under *AACT-Gal-4*. Unfortunately, the expression pattern of *AACT-Gal-4* is not specific for CA and indeed is similar to that of *Aug-21-Gal-4*. It is also expressed in a sunset of central neurons, limiting its usefulness for driving CA-specific expression.

In an attempt to identify location of ETHR expressing adult brain neurons, ETHRs were silenced in the *fru* neurons by using a *fru* specific Gal-4 line. Elevated male-male courtship after ETHR-RNAi in *fru* neurons indicates ETHR signaling is one of the downstream events specified by *fru* expression that coordinates male-specific sexual behaviors. It is still unclear if regulation of male-male courtship by ETHRs occurs via a combination of *fru* neurons and CA or is due to *fru* neurons only. Further work is needed to clarify precisely how ETHRs regulate male courtship behavior. Future experiments should focus on the CA specific Gal-4 driven ETHR-RNAi.

Misexpression of various genes elevates male-male courtship in *Drosophila* (Zhang et al. 1995; Villella et al. 1996; Demir et al. 2005; Billetert et al. 2006; Ganter, Walton et al. 2007). In an attempt to find previously known male-courtship related genes and new genes regulating male-male courtship, transcriptome analysis was done on ETHR-RNAi flies. Transcriptome analysis of three samples, CA, head and whole flies, using RNAseq on Illumina platform shows differential expression of genes that could be involved in male courtship, reproduction, axon guidance and a large number of genes involved in chromatin organization. Differential expression of chromatin organization

genes and clustering of genes suggests involvement of ETHRs in chromatin organization and possible mechanisms for regulating male courtship behavior. CA transcriptome analysis revealed the presence of male accessory gland specific genes in the CA, indicating a different function for these genes at a new location. JH related genes were differentially expressed, indicating increase in JH levels.

The outcome of transcriptome analysis allows us to generate a hypothesis to further investigate the mechanisms underlying male-male courtship resulting from ETHR-RNAi. I hypothesize that ETH plays an allatostatic function in *Drosophila* males, such that disruption of ETH signaling results in increased JH production. A model was developed based on the hypothesis. Differentially expressed gene analysis shows role of ETHRs in chromatin organization and JH biosynthesis regulation. ETHR-RNAi alters chromatin organization and increases JH levels, which affects sensory system to increase male-male courtship. The effect of JH in adult females has been extensively investigated, but less is known about functions of JH in males.

One major challenge that most biologists might face today is the limited background in computer programming and bioinformatics analysis. RNAseq data demands for bioinformatics analysis at the initial processing stage make it difficult for biologists to function independently of bioinformaticians. RNAseq, being a new technique, lacks a standard procedure for data analysis and making it challenging for researchers to analyze the massive amount of data obtained from the analysis. Increased usage of this technique and recent advances in technologies and online available user-

friendly tools like www.flymine.org enables researchers to generate new hypotheses based on differentially expressed genes.

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