Juvenile Hormone esterase is a conserved regulator of starvation-induced behavior

by

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Abstract

Although feeding behavior is a matter of life and death for animals, the genetic factors that control it remain poorly understood. We have identified a novel regulator of hunger-induced behavior through comparison of transcriptomic changes in the fruit fly *Drosophila melanogaster* and the yellow fever mosquito *Aedes aegypti*. Head mRNA from each insect was sequenced at a roughly equivalent level of starvation. Using data gleaned from the protein orthology database OrthoDB, we looked for gene pairs in which both *A. aegypti* and *D. melanogaster* orthologs were significantly regulated by starvation. This identified *Juvenile Hormone esterase (Jhe)* as a possible modulator of hunger-induced behavior. Pan-neuronal knockdown of *Jhe* resulted in increased food consumption and caused enhancement of starvation-induced sleep suppression in *Drosophila*. These behavioral phenotypes were not caused by a developmental or metabolic defects, and were reproduced by feeding adult *Drosophila* methoprene, a synthetic Juvenile Hormone analog. Application of precocene I, an inhibitor of Juvenile Hormone biosynthesis, reversed the phenotype. Our analysis suggests that *Jhe* (and Juvenile Hormone by extension) is a novel and biologically relevant regulator of hunger-induced behavior.

Preface

This work was conducted at the University of British Columbia's Life Sciences Center by Jeff Stafford and Dr. Michael Gordon. Alex Keene's (University of Nevada at Reno, Department of Biology) laboratory performed RNA sequencing of the *Drosophila melanogaster* samples used in this study, providing raw FASTQ read files for alignment and bioinformatic analysis. Carl Lowenberger (Simon Fraser University, Department of Biological Sciences) generously reared and donated the *Aedes aegypti* mosquitoes used in this study. Sean Jewell (University of British Columbia, Department of Statistics) devised and coded the vast majority of the Java statistical simulation used to verify the ortholog results (see Section 2.7). I made a number of modifications to the simulation, adding the ability to use two α values and a Gradle build script to allow compilation of a runnable "fat JAR" from the simulation source code. Michael Gordon oversaw this research project and provided funding. All other work and experiments not specifically listed here were performed by Jeff Stafford.

Table of Contents

Abstract . . . . . **ii**

Preface . . . . . **iii**

Table of Contents . . . . . **iv**

List of Tables . . . . . **vii**

List of Figures . . . . . **viii**

List of Abbreviations . . . . . **ix**

Acknowledgments . . . . . **xiii**

1 Introduction **1**

1.1 Gustation 1

1.2 Selection of experimental animals 4

1.2.1 *Drosophila melanogaster* 6

1.2.1.1 The GAL4/UAS System 6

1.2.1.2 RNA Interference (RNAi) 7

1.2.2 *Aedes aegypti* 7

1.2.3 *Tribolium castaneum* 8

1.3 Orthology as a means of transcriptomic comparison 8

1.4 Insect feeding behaviors 10

1.5 Juvenile Hormone 11

1.5.1 Synthesis of Juvenile Hormone derivatives 13

1.5.1.1 Regulation of Juvenile Hormone synthesis . . . 14
1.5.2 Juvenile Hormone metabolism in the hemolymph 15
1.5.2.1 *Takeout* 15
1.5.2.2 *Juvenile Hormone esterase* 16
1.5.2.3 *Juvenile Hormone epoxide hydrolase* 17
1.5.3 Juvenile Hormone signalling 18
1.5.3.1 *Methoprene-tolerant* and *Germ-cell Expressed* . 18
1.5.3.2 *Ultraspiracle* 19
1.5.4 Known roles and effects of the Juvenile Hormones 20

2 Methods 22
2.1 Insect husbandry 22
2.2 Tissue sugar quantification 22
2.3 RNA sample preparation 24
2.4 cDNA library preparation and sequencing 24
2.5 RNA sequencing analysis pipeline 25
2.6 Identification of conserved transcriptomic changes 25
2.7 Validation of ortholog results 26
2.8 Methoprene and Precocene I feeding 27
2.9 Measurement of *Drosophila* food intake 28
2.10 Measurement of *Drosophila* sleep 29
2.11 Measurement of *Drosophila* starvation sensitivity 29
2.12 `actmon` R package 29
2.13 Plotting and statistics 30

3 Results 31
3.1 Characterization of insect hunger 31
3.1.1 Determination of *Drosophila* starvation state 31
3.1.2 Determination of *Aedes* starvation state 33
3.2 RNA sequencing reveals conserved transcriptomic changes 33
3.2.1 Identification of conserved transcriptomic changes 35
3.2.2 Validation of ortholog results 36
3.2.3 *Jhe* is regulated significantly by hunger 38

3.3 *Jhe* is necessary for proper feeding behavior 40
3.4 *Jhe* knockdown increases starvation-induced sleep suppression . . 44
3.5 *Jhe* knockdown does not increase starvation sensitivity 45
3.6 Methoprene feeding results in a *Jhe*-like phenotype 48
3.7 Precocene I rescues *Jhe* knockdown 51

4 Discussion 54
4.1 Regulation of *Jhe* is evolutionarily conserved 54
4.2 *Jhe* affects sleep and feeding in adult *Drosophila* 56
4.3 *Jhe* exerts its effects through Juvenile Hormone metabolism . . . 56
4.4 Future work and directions 57
4.5 Final remarks 59

Bibliography 60

A Supporting Materials 69
A.1 `GCaMP_4D` 69
A.2 `fly_tracker` 71

List of Tables

Table 2.1 Key to *Drosophila* genotypes used in figures. 23

List of Figures

Figure 1.1 Measurement of *Tribolium* sugar levels 9
Figure 1.2 The Juvenile Hormones 12

Figure 3.1 Quantification of insect sugar levels 32
Figure 3.2 Overview of RNA sequencing results 34
Figure 3.3 Orthologous gene-pairs significantly regulated by starvation . 37
Figure 3.4 Ortholog simulation results 39
Figure 3.5 JH-related genes are downregulated in multiple forms of nutrient deprivation 41
Figure 3.6 *Jhe* is a modulator of feeding behavior 42
Figure 3.7 *Jhe's* phenotype is not a developmental defect 43
Figure 3.8 *Jhe's* phenotype is not an off-target effect 44
Figure 3.9 *Jhe* knockdown alters *Drosophila* activity 46
Figure 3.10 *Jhe* knockdown increases starvation-induced sleep suppression 47
Figure 3.11 *Jhe* knockdown does not affect starvation sensitivity 49
Figure 3.12 Methoprene alters *Drosophila* activity in a *Jhe*-like manner . . 50
Figure 3.13 Methoprene increases starvation-induced sleep suppression . . 52
Figure 3.14 Precocene I rescues *Jhe* knockdown 53

Figure A.1 Sample `GCaMP_4D` output 70
Figure A.2 Sample `fly_tracker` output 72

List of Abbreviations

α	The probability of any given gene being regulated in an organism
ANOVA	Analysis of Variance
BAM	Binary Alignment Format
BDGP	Berkeley *Drosophila* Genome Project
BRH	Best Reciprocal Hits
bp	Base pair/s
C	Degrees Celsius
CDF	Cumulative Distribution Function
cDNA	Complementary DNA
CNS	Central nervous system
CO_2	Carbon Dioxide
csv	Comma-separated values
DAM	Trikinetics' *Drosophila* Activity Monitor
d	Day/s
dH_2O	Distilled water
dILP	*Drosophila* Insulin-like Peptide

DNA	Deoxyribonucleic acid
FA	Farnesoic acid
FASTQ	FASTQ read format
g	Gravities
GAL4	The *Saccharmyces cerevisiae* GAL4 protein
gce	*Germ-cell Expressed*
GD	VDRC GD Transgenic RNAi Library
GFP	Green Fluorescent Protein
GPS	Graduate and Postdoctoral Studies
GTF	Gene Transfer Format 2
h	Hour/s
JAR	Java archive
JH	Juvenile Hormone
jhamt	*Juvenile Hormone acid methyltransferase*
JHB3	Juvenile Hormone III bisepoxide
hJHBP	Hemolymph Juvenile Hormone binding protein
Jhe	*Juvenile Hormone esterase*
Jheh	*Juvenile Hormone epoxide hydrolase*
k	Number of significantly regulated orthologous gene pairs
KCl	Potassium Chloride
KK	VDRC KK Transgenic RNAi Library
Met	*Methoprene-tolerant*

MF	Methyl farnesoate
min	Minute/s
mL	Milliliter/s
mm	Millimeter/s
mM	Millimolar
mRNA	Messenger RNA
NaCl	Sodium Chloride
OrthoDB	OrthoDB7, a heirachical catalog of animal, fungal, and bacterial orthologs
PCR	Polymerase Chain Reaction
PMF	Probability Mass Function
R	The R programmming language
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RNAi	RNA interference
S4	The R language's S4 object-oriented programming system
shRNA	Small hairpin RNA
sNPF	Short Neuropeptide F
SWIPE	The SWIPE protein alignment algorithm
TARGET	Temporal And Regional Gene Expression Targeting system
Tris-HCl	Tris (hydroxymethyl) Aminomethane Hydrochloride
To	*Takeout*

µg	Microgram/s
µL	Microliter/s
UAS	Upstream Activating Sequence, targeted by GAL4
UBC	The University of British Columbia
usp	*Ultraspiracle*
VDRC	Vienna *Drosophila* Resource Center
VL20	Bloomington VALIUM20 RNAi Library
w/v	Percent weight / volume

Acknowledgments

Before diving into the study head-on, I wanted to take a moment to thank all of the people who helped me get where I am today. First and foremost, I'd like to thank my collaborators. Carl Lowenberger raised and supplied the mosquitoes used for this study, making it possible to perform the RNA-Seq experiments for that organism. Alex's Keene's *Drosophila* RNA-Seq datasets were instrumental as well, and enabled us to much more accurately quantify several transcriptomic changes than would otherwise have been possible. This study would not have been possible without WestGrid and Compute Canada's generous donation of computer time for use in RNA-Seq alignment and quantification. Brian de Alwis's LaTeX template made writing this thesis far easier than it otherwise would have been. I'd also like to take a moment to thank the members of my committee- it was refreshing to hear new viewpoints, and your input was greatly appreciated. Most importantly, I want to thank my supervisor, Mike Gordon, for giving me the chance to start here and the freedom to take my research in the direction I wanted. Last but not least, I'd like to thank my friends, roommates, and family. Without you to keep me sane, I never would have made it this far.

Chapter 1

Introduction

1.1 Gustation

The gustatory (taste) system is an ideal model with which to study larger processes occurring throughout the brain as a whole. Compared with other sensory modalities, gustation is relatively simple. Humans have five tastes: sweet, salty, sour, bitter, and umami. In contrast, most people would be hard pressed to tell you how many smells there were or describe what might comprise different types of touch. Taste is a discrete stimulus that is easy to quantify. Taste processing in the brain is considered relatively simple as well. The olfactory system uses a combinatorial code to encode stimuli, with the same areas of the brain activated in different patterns to represent different smells (Semmelhack and Wang, 2009). By contrast, the dominant theory is that gustatory inputs are processed as a labelled line: each different taste is carried to the brain along different nerves and is processed in different regions (Yarmolinsky et al., 2009). Gustation is also thought to be a hardwired system. Whereas responses to olfactory stimuli can be learned by association, taste evokes stereotyped, reflexive behavior (Yarmolinsky et al., 2009). When an experimentally-induced change causes gustatory information to be processed differently, gustatory behavior will often change as a result. Because of this, gustatory behavior is an ideal model with which to study the link between genes and behavior.

Much is known about how gustatory information is detected at the sensory

level. Despite this, relatively little is known about how this information is processed in higher brain centers and used to effect changes on feeding-related behavior. Although several neurons responsible for directly controlling feeding behavior have been identified, these neurons do not directly synapse with the neurons capable of detecting gustatory stimuli (Gordon and Scott, 2009). Very few second-order taste neurons have been identified, and the many of those that have do not integrate input from internal stimuli like hunger state (Flood et al., 2013; Chu et al., 2014; Kain and Dahanukar, 2015). Although the neurons and mechanisms controlling complex gustatory behaviors have been discovered (such as taste-independent calorie sensing), their exact means of effecting changes in behavior remain unclear (Miyamoto et al., 2012; Stafford et al., 2012; Dus et al., 2013). Most, if not all, of these gustatory sensory pathways and behaviors are known to be influenced by starvation.

Starvation is a fundamental physiological state shared by all animals, yet it remains poorly understood. Starved animals change behavior dramatically- hungry *Drosophila* eat more, rapidly develop a preference for high-calorie foods, and suppress sleep (Stafford et al., 2012; Keene et al., 2010). A large number of genetic and hormonal changes occur during starvation as well. Despite this information, it remains relatively unknown which genes and signaling pathways are necessary and sufficient to elicit a starvation-like behavioral state in animals. The goal of this study was to discover a gene or signaling pathway capable of inducing such a behavioral change. Any novel behavioral regulator identified would prove a tantalizing target for further research examining how gustatory behaviors are controlled by the brain.

Before this study, it was known that a large number of transcriptional changes occurred in an animal during starvation (Farhadian et al., 2012; Fujikawa et al., 2009). These transcriptional changes seemed likely to control starvation-related behavior, as the process of transcription acts on a similar timescale to starvation itself. The most straightforward means of measuring these changes would be to simply run a set of microarrays of RNA extracted from the head of a model organism like *Drosophila melanogaster* when it is hungry. Examining *Drosophila's* head transcriptome is a simple and effective means of studying neuronal changes in expression- with the exception of the ventral nerve cord, it captures the entire

central nervous system (CNS) of an animal, along with important sensory organs like the eyes, antennae, and labella (mouthparts). This approach would capture any transcriptional changes in that occurred in the brain and sensory tissues during starvation. However, this method has been tried twice before. Microarrays peformed in this manner did not identify any previously unstudied behavioral regulators (Farhadian et al., 2012; Fujikawa et al., 2009). Despite these results, it seemed likely that there were still genes of interest that could be discovered using a more advanced approach.

Advancements in technology now allow for much more accurate and sensitive quantification of gene expression changes, with a much greater dynamic range (Wang et al., 2009). Capitalizing on this fact, this study utilized high-throughput mRNA sequencing instead of microarrays for this study. For instance, one commercial RNA-Seq platform is advertised as detecting the same number of differentially expressed genes as a typical microarray with a mere 2 million reads (Illumina, 2011). I aimed to sequence to a depth of 35-60 million reads, a much more exact reading of RNA abundance than was possible previously. Additionally, RNA sequencing is able to perform accurate quantification of mRNA abundance across a much larger dynamic range. Microarrays are notoriously poor at quantifying weakly or very highly expressed transcripts (Wang et al., 2009). Accurate quantification of weakly expressed genes was of particular concern for this study. Many genes with a large impact on behavior, including sex specification genes like *fruitless* or hormones like the *Drosophila* Insulin-like Peptide family, are expressed in extremely spatially restricted patterns, with expression limited to only a handful of cells. RNA sequencing offers an effective, reliable means of accurately quantifying these types of transcripts.

Screening *Drosophila* or any other animal for behavioral phenotypes is extremely slow and suffers from highly variable results. Behavioral experiments require numerous genetic and environmental controls, and promising results need to be repeated several times on several different days before they can be accepted. Even screening less than a hundred RNAi knockdown lines can take months, depending on the behavioral assay used. Any means of refining a large list of differentially expressed genes down to a handful of promising candidates could potentially save an inordinate amount of research time when screening for potential behav-

ioral regulators. As a result, I decided to employ a new means of improving our our chances of finding a novel regulator of hunger-induced behavior. I decided that studying hunger-induced changes in two separate, evolutionarily divergent insects would offer a good chance of highlighting potentially interesting genes. The logic behind this decision was that any transcriptomic changes conserved between the organisms would be the most important to an animal's survival, as regulation of these genes had been maintained across millions of years of evolution. Of course, this approach raises a number of important questions. What experimental animals would prove the best candidates for study? What would constitute a "conserved transcriptomic change?" How would it be established that the animals were in a similar physiological state before sequencing?

1.2 Selection of experimental animals

As I previously indicated in Section 1.1, insects were an ideal target for this study as it is possible to sequence the entire head in a single sample, encapsulating most of the CNS and all associated sensory tissues. Other advantages offered by insects over mammalian and other genetic systems include extremely fast generation times, a large number of available genetic tools and behavioral assays, and pre-existing expertise within our research group. In addition, the vast majority of insect genes have human homologs, and even genes without mammalian equivalents often have important commercial applications (possibly having implications for pesticide development or rearing of economically vital species like honeybees).

For the purposes of this study, there were several key requirements for a species to be chosen as a research subject:

1. **The species must have a sequenced genome.**

   Although *de novo* transcriptome alignment and assembly is now attainable with the aid of bioinformatics tools like Trans-ABySS and Cufflinks, it adds an additional layer of complexity to the analysis and most gene and transcript annotations would have to be predicted (Robertson et al., 2010; Trapnell et al., 2010). Using an animal with a sequenced genome would provide immediate results and would avoid the potential risk of falsely predicted

gene models.

2. **The species chosen must have stable gene annotations**

   Because this study would rely heavily on externally derived orthology annotations, the stability of gene annotations would become extremely important. Using an organism with unstable, frequently changed annotations would make it significantly more difficult to match our sequencing data with external data.

3. **Each species must be readily obtainable and easy to raise.**

   The sample preparation protocol used in this study required a significant amount of tissue. Preliminary experiments indicated that I would need roughly 100 insect heads per sample in order to obtain enough RNA for cDNA library preparation and sequencing. To obtain enough tissue for sequencing, I would need an insect species that survived well in a lab environment and could be bred in batches of at least 600 individuals, or enough samples for 3 biological replicates of each condition.

4. **Any species chosen must be of general scientific, medical, or commercial relevance.**

   Identifying a novel regulator of hunger-induced behavior would be much more interesting and impactful if I would be able to demonstrate that it controls phenotypes in a major research model organism or species that people otherwise deal with on a regular basis.

5. **The group of species chosen should not be too closely related to each other.**

   Comparing gene regulation between distinct species allows us to argue that any genes with conserved changes are likely more important to an animal's survival. The rationale for this is that the regulatory patterns of those genes during starvation has been maintained across millions of years of evolution. More evolutionarily distant species make this argument more compelling- their genomes and accompanying regulatory elements would have diverged significantly, and only a small number of the most critical gene regulatory

patterns would be likely to have been conserved between them. In contrast, a set of extremely closely related species (like members of the *Drosophila* genus) would make a poor choice, as they would be expected to share a large number of gene regulatory changes, potentially masking interesting results.

Using the criteria outlined here, we identified three insect species of potential interest to our study: the fruit fly *Drosophila melanogaster*, the yellow fever mosquito *Aedes aegypti*, and the red flour beetle *Tribolium castaneum*. The individual factors that lead to these organisms' inclusion or exclusion in the final study are discussed below.

1.2.1 *Drosophila melanogaster*

Drosophila melanogaster was the most obvious choice of species for our study. Fruit flies have been used in scientific studies for over a hundred years, and a large number of genetic tools and logistical resources exist for this organism. Both current and archived versions of the reference genome and its associated annotation are accessible through both Ensembl and FlyBase, with automated retrieval of bioinformatics data made possible through APIs like BioMart (Flicek et al., 2014; dos Santos et al., 2015; Durinck et al., 2005). *Drosophila* is extremely well studied, with experimental information and electronic annotations available for every gene. Two genetic tools made *Drosophila* of particular interest to our study as well: the GAL4/UAS binary expression system and the wide availability of RNA interference lines. These tools would allow us to knock down nearly any gene of interest at any time and place. The relative ease of genetic manipulation and well-characterized behaviors of *Drosophila* meant that the species would be an ideal tool with which to screen genes for roles in behavior.

1.2.1.1 The GAL4/UAS System

The GAL4/UAS system provides an simple and effective system for controlling the expression of transgenic genes. GAL4, a transcription factor from *Saccharomyces cerevisiae*, induces expression of sequences preceded by an Upstream Activating Sequence (UAS) (Brand and Perrimon, 1993). In one expression strategy, the gene encoding GAL4 is directly inserted within a sequence of interest. GAL4 is then

expressed in the same tissues its flanking sequences are expressed in, driving expression of UAS reporter/effector constructs (Brand and Perrimon, 1993). Generally, *Drosophila* lines with a GAL4 "driver" and UAS "reporter" are created and maintained separately. The same GAL4 line can then be used to drive expression of multiple different reporters in the same target tissue, depending on what line it is crossed with. Conversely, a researcher can test the effects of a particular UAS reporter in multiple tissues by crossing a single UAS with different GAL4 lines (Duffy, 2002). GAL4 can also be reversibly inactivated by the repressor protein GAL80. A temperature-sensitive version of GAL80 has been created as well (hereafter referred to as GAL80^{ts}), allowing a researcher to temporally restrict expression of UAS targets to time periods where *Drosophila* has been maintained above a "restrictive temperature" (typically 29C) (McGuire et al., 2004). In this study, the GAL4/UAS/GAL80^{ts} system allowed expression of effector genes like RNAi constructs at virtually any time and place.

1.2.1.2 RNA Interference (RNAi)

RNA interference, or RNAi, utilizes transgenic expression of short RNA sequences to induce targeted destruction of mRNA in live tissues. In *Drosophila*, RNAi is typically performed by UAS expression of a small hairpin or double-stranded RNA complementary to a target sequence. These RNA molecules are then cleaved by the RNA processing enzyme Dicer and recruited to the RNA-induced silencing complex, which destroys RNAs complementary to the original shRNA sequence (Wang et al., 2006). Combined with the wide availability of UAS-RNAi lines for nearly every *Drosophila* gene, RNA interference is a powerful tool with which to study the effects of knocking down transcriptomic targets.

1.2.2 *Aedes aegypti*

The mosquito *Aedes aegypti* is a vector for yellow fever, dengue fever, and chikungunya. This mosquito species made an excellent candidate for this project as it had already been widely studied and we were able to obtain specimens relatively easily. In addition, the online Vectorbase database has a multitude of *Aedes aegypti*-related datasets available for use, making it extremely easy to obtain the ref-

erence genome and genebuilds necessary for any bioinformatics analysis (Giraldo-Calderon et al., 2015). The only complicating factor for my analysis was the fact that female mosquitoes must consume blood in order to reproduce. To avoid any potential pitfalls associated with this extra feeding modality, I chose to sequence male mosquitoes and flies. This strategy would allow us to strictly study the genetic changes induced by food deprivation (instead of food and blood deprivation in mosquitoes).

1.2.3 *Tribolium castaneum*

The red flour beetle, *Tribolium castaneum* was another strong candidate species for this study. This insect is a major pest of stored grain products and has been widely used as an insect developmental model. Although *T. castaneum* has a sequenced genome and otherwise met all of the requirements set out in Section 1.2, several preliminary experiments indicated that this insect might not be the right choice for this study. *Tribolium* beetles are extremely starvation-resistant, and can survive over a week without any food or water. When its tissue sugar levels were measured over time during starvation, these sugar levels did not follow the pattern observed in adult *D. melanogaster* or *A. aegypti*. In flies and mosquitoes, sugar levels dropped rapidly, eventually plateauing at a "base level" before death (see Figure 3.1). The sugar levels of *Tribolium* beetles did not follow this pattern, with glucose levels dropping rapidly (see Figure 1.1), then slowly rising over time until death. I hypothesize that this might be caused by metabolism of energy stores not quantified during our experiment, like starch (the food used to rear *Tribolium* is simply flour and yeast mixed together). Due to these differences, I felt that the red flour beetle was not a good candidate for transcriptomic comparison with *Drosophila*.

1.3 Orthology as a means of transcriptomic comparison

One of the major stumbling blocks for this study was attempting to determine what constitutes a "conserved transcriptomic change." Analyzing the transcriptomes of two separate species would be fruitless without a biologically meaningful method of comparing them. In order to accurately compare transcriptomes, I used the concept of orthology to pair species' transcriptomes. By definition, orthologs are

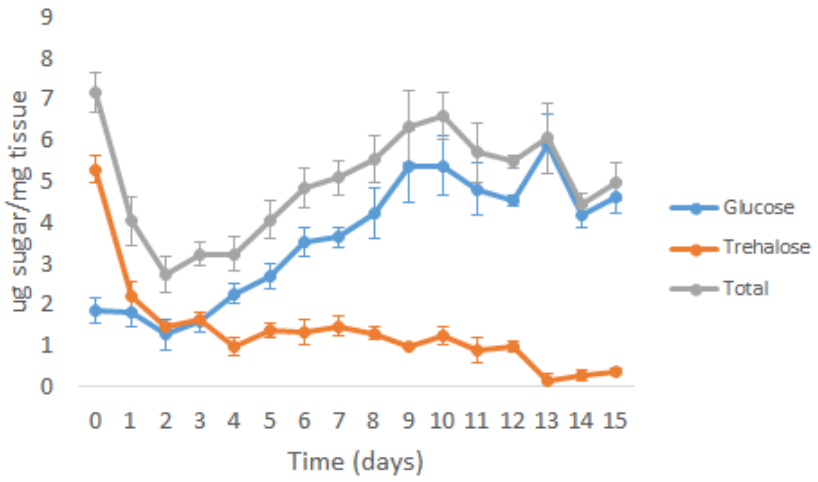

Figure 1.1: Measurement of *Tribolium* sugar levels.
Glucose, trehalose, and total sugar levels quantified from *Tribolium castaneum* beetles during starvation. n = 4-8 replicates of individual male beetles. Error bars represent standard error of the mean. Data for the equivalent experiment in *D. melanogaster* and *A. aegypti* are presented in Figure 3.1.

genes in separate species that diverged from a common ancestor gene as part of the process of speciation. This means that orthologs will typically share significant sequence identity, and possibly a similar biological function as well. The basic biological definition above sets no strict criteria as to how to determine what constitutes an ortholog. Although manual annotation of genes as orthologs is effective, there are no guarantees that all researchers involved utilized the exact same methodology. Additionally, manual annotation of orthologs will be biased towards commonly used experimental models. Species that have been studied more will have a larger number of annotated orthologs.

To address this concern, I turned to a computational definition of orthology instead, in this case the OrthoDB7 database created by Waterhouse et al. (2013). OrthoDB7's definition uses the amino acid sequence of each gene's longest transcript to define genes as orthologs based on their similarity across species (Waterhouse et al., 2013). This avoids the pitfalls of manual annotation (where the most-studied organisms would have the most data). Since each species' genome in the OrthoDB7 database has been sequenced, each species will have a standardized

analysis done in an identical manner to that of other species'. Furthermore, as this method relies only upon sequence identity, it will be able to identify potential orthologs for every gene in every genome in the database. Using this definition, I was able to successfully match up and compare the transcriptomes of *D. melanogaster* and *A. aegypti* using the protocol described in Section 2.6. The results and validation of this approach are described in Sections 2.7, 3.2.1 and 3.2.2.

1.4 Insect feeding behaviors

The goal of this project was to identify a novel regulator of starvation-induced behavior. Once we identified a conserved set of genes up- or downregulated by starvation, I planned to screen those genes for effects on behavior. If a gene was downregulated by starvation, this change could be reproduced using RNAi knockdown in fed flies. If a gene was upregulated by starvation, it would be possible to prevent the gene's upregulation using RNAi knockdown in starved flies. Either scenario allowed us to assess if a gene's regulation resulted in starvation-like behavior.

I reasoned that any gene regulated by starvation would likely serve to increase an animal's resistance to starvation, either by directly manipulating metabolism or modifying feeding/foraging behaviors. As a result, there are several different types of feeding behavior that I chose to quantify. The first is a simple assessment of the raw volume of food consumed. Like any other animal, flies eat more after starvation. Another behavioral paradigm chosen for study was caloric sensing. Flies are capable of sensing the calorie content of foods, and adjusting their food intake accordingly (Stafford et al., 2012). Genes' effect on these two behaviors was assessed using a modified version of the CAFE assay (Ja et al., 2007). A description of the protocol used is found in Section 2.9.

Sleep is also known to be regulated by internal metabolic state. Previous work performed by Keene et al. (2010) indicated that starved flies will suppress sleep in order to forage for food. This is manifested in a major decrease in sleep after 12 hours of starvation. Although this phenomenon is known to be modulated by the *clk/cyc* group of circadian neurons, the genes and neural circuit responsible for directly controlling this behavior remain unknown (Keene et al., 2010). This sleep

suppression phenotype can be assessed using the *Drosophila* Activity Monitor in the manner described in Section 2.10.

1.5 Juvenile Hormone

One class of genes known to be heavily implicated in feeding-related behaviors are hormones and their respective hormone-processing enzymes. This study identified Juvenile Hormone as a regulator of starvation-related behavior. To aid in interpretation of experimental results, a comprehensive review of Juvenile Hormone is provided here.

Juvenile Hormone (JH) is an insect developmental cue, acting in opposition to 20-hydroxyecdysone (ecdysone) to regulate the growth from larva to adult. A short pulse of ecdysone initiates each major developmental transition in insects, whether it be molting or metamorphosis. JH acts to oppose ecydone, and its absence in the final instar of insect development allows ecdysone to trigger metamorphosis. As a result, the most well characterized function of JH is it's ability to delay metamorphosis, either adding extra larval instars in moths and beetles, or preventing cellular differentiation and triggering apoptosis in higher diptera like *Drosophila* (Bernardo and Dubrovsky, 2012). Although ecdysone and JH are both steroid hormones that act on similar processes, JH is less well studied, and its exact modes of action remain somewhat unclear. JH appears to have a wide ranging set of effects in addition to its metabolic role, including sexual behavior, pheromone production, caste determination, diapause, migration, and the synthesis of gonadal proteins (Bernardo and Dubrovsky, 2012).

Part of Juvenile Hormone's complexity arises from the fact that JH is not actually a single hormone, but a collection of extremely similar steroids that each have biological activity. Different species of insects secrete different Juvenile Hormones. Lepidopteran insects (butterflies and moths) secrete five Juvenile Hormones: JH 0, JH I, JH II, JH III, and 4-methyl JH I. *Drosophila*, on the other hand, secrete three different Juvenile Hormone derivatives: JH III, Juvenile Hormone III Bisepoxide (JHB3), and methyl farnesoate (MF). JHB3 is only secreted in higher Diptera like Drosophilid flies. Heteropteran insects (a suborder of insects that includes bedbugs and water striders) secrete an additional JH: Juvenile Hormone III

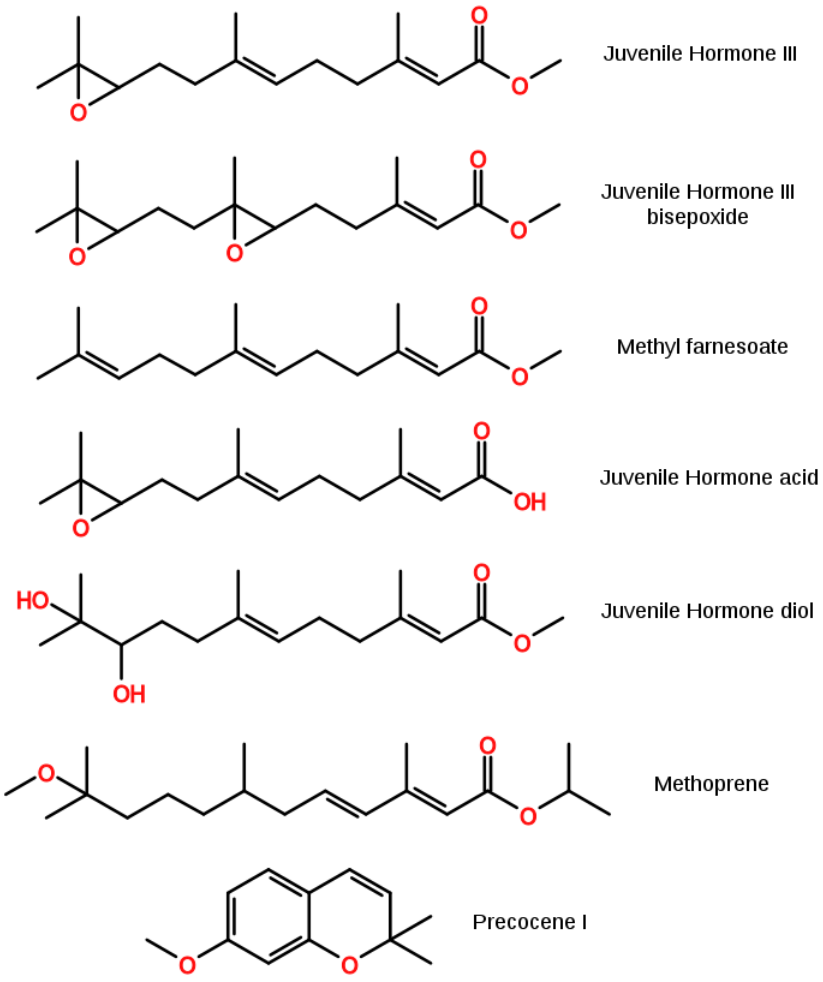

Figure 1.2: The Juvenile Hormones.
Chemical structures of the Juvenile Hormones found in *Drosophila*, breakdown products of Juvenile Hormone III, and synthetic Juvenile Hormone agonists/antagonists used in this study.

Skipped Bisepoxide (Noriega, 2014). For simplicity's sake (and since this study focuses so heavily on *Drosophila*), this introduction will focus on JH III, JHB3, and MF.

1.5.1 Synthesis of Juvenile Hormone derivatives

Juvenile Hormones are synthesized in the corpora allata, an endocrine gland located next to the esophagus in *Drosophila*. This synthesis pathway for JH is part of the larger mevalonate pathway found throughout the animal kingdom (responsible for cholesterol synthesis in mammals) (Noriega, 2014). Briefly, multiple units of Acetyl-CoA are converted into farnesyl pyrophosphate by nine enzymes in the mevalonate pathway. The Juvenile Hormone biosynthetic pathway diverges here, and farneysl pyrophosphate is converted to farnesol by farnesyl pyrophosphatase. Farnesyl dehydrogenase then converts farnesol to farnesoic acid in a two-step reaction. In insects that produce only JH III, farnesoic acid is used to produce methyl farnesoate by Juvenile Hormone acid methyltransferase (JHAMT). Methyl farnesoate is processed by Methyl farnesoate epoxidase (a cytochrome P450 CYP15 expoxidase) to produce JH III (Noriega, 2014).

Unfortunately, the last two steps of JH synthesis in *Drosophila* are not as clear. Although farnesoic acid (FA) is the common precursor for JH III, JHB3, and MF, there are potentially mutiple synthesis pathways for each, and interconversion between these JH derivatives does occur (Wen et al., 2015). The first major difference in the synthesis pathway is the lack of a clear CYP15 epoxidase in flies - the *Drosophila* CYP15 sequence has evolved so signifcantly that it is no longer considered a member of the CYP15 gene family. Wen et al. (2015) proposed that CYP6G2 may function in a similar role to CYP15s due to CYP6G2's expression in the *Drosophila* corpora allata, but admit that there is no biochemical evidence for this function either *in vitro* or *in vivo* . It is thought that whatever enzyme catalyzes this step (CYP6G2 or an unknown cytochrome P450 expoxidase) produces two products: 10,11 epoxifarnesoic acid or 6,7;10,11 epoxifarnesoic acid, acting as a precursor for JH III or JHB3, respectively (Wen et al., 2015). The higher abundance of JHB3 in *Drosophila* may due to a preference of CYP6G2 for 6,7;10,11 epoxifarnesoic acid as a product (Wen et al., 2015).

Another major difference in *Drosophila's* synthesis pathway is the role of JHAMT. Wen et al. (2015) found that overexpression or mutation of JHAMT (normally responsible for producing MF from FA in other insects), did not affect the rate of JH III or MF synthesis. Unexpectedly, only the amount of JHB3 changed.

This indicates that JHAMT is primarily responsible for synthesizing JHB3 from 6,7;10,11 epoxifarnesoic acid, unlike the role it plays in producing MF in other insects (Wen et al., 2015).

This is the extent of current knowledge of Juvenile Hormone synthesis in *Drosophila*. The methyltransferase that converts FA to MF in the corpora allata remains unknown. The methyltranferase responsible for producing JH III from 10,11 epoxifarnesoic acid has not been identified either.Wen et al. (2015) found evidence for interconversion of MF and JH III into JHB3 in the hemolymph of flies, presumably by another cytochrome P450 expoxidase that has yet to be identified.

1.5.1.1 Regulation of Juvenile Hormone synthesis

In mammals, the mevalonate synthesis pathway is regulated by sterol molecules through Sterol Regulatory Element Binding Protein (SREBP). This is not the case in insects, as homologs for the SREBP and SREBP-2 proteins do not exist in these animals (Goodman and Cusson, 2012). Instead, it is thought that JHs directly regulate their own synthesis, and have been shown to have a regulatory effect on mevalonate synthesis enzymes in tissues outside the corpora allata (Goodman and Cusson, 2012).

Perhaps more strikingly, it has been reported that Short Neuropeptide F (sNPF) may be involved in regulating Juvenile Hormone biosynthesis. While working on the silkworm *Bombyx mori*, two putative sNPF receptors were identified in the corpora allata. When *B. mori* sNPF peptides were assayed for effects on JH synthesis *in vitro*, they had a strong inhibitory effect (Goodman and Cusson, 2012). This is especially interesting, as sNPF has previously been shown to be heavily implicated in starvation-related behaviors such as odor-driven food search and control of food intake in *Drosophila melanogaster* (Hong et al., 2012; Root et al., 2011). The corpora allata is heavily innervated by higher brain centers, including nerves that originate in the subesophageal ganglion, a region of the brain that plays a major role in feeding behaviors (Goodman and Cusson, 2012). Although this is pure speculation, it may be the case that synthesis of JH-related compounds may be regulated by higher brain centers through neuropeptides like sNPF. Although this

seems to be a likely explanation for these phenomena, there is no direct experimental evidence for this.

1.5.2 Juvenile Hormone metabolism in the hemolymph

Once synthesized by the corpora allata, JH III, JHB3, and MF are secreted into the hemolymph. Once there, these JH hormone derivatives are capable of being acted upon by a number of different proteins, including several unknown enzymes previously mentioned in Section 1.5.1. One of these proteins, Takeout (TO), is thought to be responsible for JH transport, whereas Juvenile Hormone esterase (JHE) and Juvenile Hormone epoxide hydrolase (JHEH) degrade it.

1.5.2.1 *Takeout*

There are many proteins in insects capable of transporting Juvenile Hormones, including both low-affinity, general binding proteins like lipophorins and specific binding proteins that bind JHs with extremely high affinity, like Takeout. Takeout belongs to a small family of hemolymph Juvenile Hormone binding proteins (hJHBPs). The *Manduca sexta* hJHBP preferentially binds JHs I, II, and III, but direct binding has never been demonstrated in *Drosophila* (Goodman and Cusson, 2012). hJHBPs are thought to have a number of important physiological roles, including transport of JHs to target sites, reducing promiscuous activity in non-target tissues, preventing JH degradation, and providing a reservoir of hormone near target tissues (Goodman and Cusson, 2012). In *Drosophila*, *takeout* mutants demonstrate increased starvation sensitivity, increased feeding, increased sensitivity of sugar-specific gustatory neurons, and increased lifespan (Meunier et al., 2007; Sarov-blat et al., 2000; Chamseddin et al., 2012).

There is evidence for hJHBP regulation by JH itself. In *Manduca sexta*, JH titers and hJHBP expression are inversely related (Goodman and Cusson, 2012). *Drosophila's Takeout* is regulated in a circadian manner, and its expression is dependent on the circadian genes *per* and *tim* (Sarov-blat et al., 2000).

1.5.2.2 *Juvenile Hormone esterase*

There are two primary pathways of JH degradation in insects- conversion of JH III to JH acid by Juvenile Hormone esterase, or conversion of JH III to JH diol by Juvenile Hormone epoxide hydrolase. Although *Drosophila* has a number of *Jhe*-like Juvenile Hormone esterases (including the *Jhe* gene duplication *Jhedup*), only JHE has been shown to readily metabolise JHs *in vitro* (Crone et al., 2007). *Drosophila's* JHE is capable of metabolising multiple forms of JH, including JH I, JH II, JH III, JHB3, and MF, but not synthetic JH analogs like methoprene, hydroprene, or kinoprene (Crone et al., 2007; Campbell et al., 1998). JHE has an extremely high affinity for JH III, with a K_m of either 1.5 μM or 89 nM for recombinant or tissue-purified protein respectively. The catalytic efficiencies were similar for both studies, with K_{cat} values of 1.0 s^{-1} and 0.6 s^{-1} (Crone et al., 2007; Campbell et al., 1998). It is clear that *Drosophila* JHE is capable of degrading all naturally occuring forms of JH, while leaving synthetic analogs like methoprene intact (synthetic JHs were designed to be as stable as possible for use as broad-spectrum insecticides).

Jhe has three annotated transcripts: Jhe-RA, Jhe-RB, and Jhe-RC. The only difference between these transcripts lies in their 5' UTR- all 3 transcripts produce an identical peptide product (Celniker et al., 2009). Data from the modENCODE project indicates that expression of *Drosophila Jhe* occurs primarily in the pupal fat body and adult head, with highest expression during the pupal stage (Celniker et al., 2009). In a manner opposite to *Takeout*, *Jhe* expression is induced by JH and suppressed by ecdysone (Kethidi et al., 2005). This corresponds to *in vivo* levels of Juvenile Hormone. Temporal expression of *Jhe* matches JH abundance during development, with expression peaks during each larval stage. JHE protein is found both within *Drosophila* is primarily found within the hemolymph, and its abundance is tightly controlled by post-translational mechanisms. Injection of recombinant JHE into *Manduca sexta* larvae demonstrated that the protein has a half-life of 1.2 to 3.6 hours depending on the amount injected, whereas control proteins of similar mass had a half-life of days (Ichinose et al., 1992). This rapid uptake and destruction of JHE protein is mediated by receptor-mediated endocytosis followed by lysosomal degradation in pericardial cells (cells that surround the

insect heart) (Ichinose et al., 1992; Bonning et al., 1997).

1.5.2.3 *Juvenile Hormone epoxide hydrolase*

Although less well studied, Juvenile Hormone epoxide hydrolase (JHEH) is the other enzyme responsible for JH degradation in insects. The relative contribution of JHE and JHEH towards JH degradation changes over *Drosophila's* lifespan. By measuring the relative amount of JH acid and JH diol (produced by JHE and JHEH respectively), Campbell et al. (1992) determined that JHEH dominates JH catabolism during the larval stage, JHE controls JH degradation in pupae, and both enzymes contribute relatively equally in the adult stage. This evidence is corroborated by two similar experiments that measured *in vitro* activity of each enzyme purified from *Drosophila melanogaster* and *Drosophila virilis* (Casas et al., 1991; Rauschenbach et al., 1995).

Drosophila has three JHEH genes: *Jheh1*, *Jheh2*, and *Jheh3*. All three appear to show relatively widespread expression, although *Jheh3* appears to show highest expression in the digestive system (Celniker et al., 2009). The *Drosophila* JHEHs have been demonstrated to metabolise JH III and JHB3 readily *in vitro*, although JHEH-containing fractions hydrolyze JH III with between three and ten times greater efficiency than JHB3 (Casas et al., 1991). Note that JHEH cannot hydrolyse methyl farnesoate, as this molecule does not contain any epoxide functional groups. JHEH's preference for JH III as a substrate may explain why JHE contributes so heavily to JH degradation in adult flies despite the greater adult expression of *Jheh* (Celniker et al., 2009). MF and JHB3 are simply far more abundant substrates in higher Dipteran (fly) larvae and adults, and JHE is capable of metabolising these with much greater efficiency (Wen et al., 2015; Yin et al., 1995; Campbell et al., 1998). If anything, JHE's contribution to JH degradation may be more important in the greater *Drosophila* genus: addition of DFP, a general esterase inhibitor, decreased the amount of JH III degradation of adult fly homogenate by more than 10 fold in *D. virilis* compared with more than 2 fold in *D. melanogaster* (Rauschenbach et al., 1995).

1.5.3 Juvenile Hormone signalling

Juvenile Hormone is known to act upon a large number of target tissues. These effects are transduced by a number of different receptors, the two most well known of which are the MET/GCE heterodimeric receptor and USP, an interaction partner of the ecdysone receptor. Other studies have suggested that JHs are capable of binding with as-yet unidentified G-protein-coupled receptors, but evidence for this is currently lacking (Goodman and Cusson, 2012).

1.5.3.1 *Methoprene-tolerant* and *Germ-cell Expressed*

Methoprene-tolerant (Met) was the first true JH receptor identified. Ethyl methane sulfonate-based mutagenesis of *D. melanogaster* produced a mutant (*Met*) that was 100 times more resistant to Juvenile Hormone III or methoprene added to food. This mutation allowed larvae to survive otherwise lethal concentrations of methoprene, did not develop methoprene-induced pseudotumors, and exhibited normal vitelligenic oocyte development in the presence of JH agonists. Importantly, *Met* did not confer any resistance to other insecticides, indicating that the gene did not encode a general insecticide resistance, but a protein product specific in its interaction with JH (Wilson and Fabian, 1986). Further analysis by Shemshedini et al. (1990) demonstrated that Met bound directly to JH III, with a dissociation constant (at which 50 percent of substrate is bound) of 6.7 nM. The *Met* mutation, on the other hand, increased this value to 38 nM. Later analyses indicated that *Met* encoded a member of the basic-helix-loop-helix-PAS family of transcriptional regulators (Wilson and Ashok, 1998).

Remarkably, a *Met* mutation that produced no transcript, Met^{27}, was able to survive to adulthood and reproduce, albeit with an 80 percent reduction in egg production (Wilson and Ashok, 1998). Since proper levels of JH signaling is critical for insect survival and reproduction, this indicated that another gene must be able to transduce JH's effects in the absence of *Met* (Wilson and Fabian, 1986; Bowers et al., 1976; Wilson and Ashok, 1998).

Germ-cell Expressed (gce), another basic-helix-loop-helix-PAS gene, was soon identified as a critical interaction partner for *Met*. Pull-down assays in *Drosophila* S2 cells indicated that MET protein bound GCE, and this binding was disrupted

by addition of either JH III or methoprene (Godlewski et al., 2006). Importantly, Met was able to form both MET-MET homodimers as well as MET-GCE heterodimers, indicating that the two receptors may be partially redundant (Godlewski et al., 2006). Although *gce* is able to substitute for *Met* expression *in vivo*, RNAi of *gce* in *Met* mutants is lethal (Baumann et al., 2010).

Both MET and GCE transduce their effects through the FTZ-F1 transcription factor. The *E75A* is a nuclear receptor gene with expression induced by JHs. Removal of FTZ-F1 via RNAi prevented *E75A* expression in response to JH application, whereas FTZ-F1 overexprssion increased it (Dubrovsky et al., 2011). This transcription factor forms heterodimers with either MET or GCE, and transgenic expression of *Manduca sexta Jhe* blocked JH-induced expression of E75A (Dubrovsky et al., 2011).

1.5.3.2 *Ultraspiracle*

Another known receptor for JH is the nuclear receptor *Ultraspiracle (Usp)*. Jones and Sharp (1997) demonstrated that USP directly binds JH III and JHB3 (but not farnesol or ecdysone), inducing a conformational change and oligomerization of USP protein, with a dissociation constant of 0.5 μM . Further work on the original study indicates that USP is also capable of binding MF in addition to JH III and MF (Jones et al., 2010). The vertebrate ortholog of USP, the retinoid X receptor, has also been shown to respond to methoprene at high concentrations (Harmon et al., 1995).

USP is both a transcription factor and an interaction partner of the ecdysone receptor EcR (Iwema et al., 2009). Goodman and Cusson (2012) proposed that USP may interact differently when JH or ecdysone are present: USP is capable of binding JH response elements in the presence of JH, ecdysone binds to USP:EcR to initiate metamorphic molting, and when both JH and ecdysone are present, both hormones are capable of binding to this receptor complex and triggering larval molting. The dual interaction of USP with both JH and ecdysone signaling may explain the interaction of these two pathways, integrating input from both hormones. It is worth noting that exogenous application of ecdysone to adult *Drosophila* is capable of inducing sleep in an EcR-dependent manner (Ishimoto and Kitamoto,

2010). This is the opposite effect of adding methoprene to adult flies (see Section 3.6), and these two seemingly antagonistic phenotypes may in fact be mediated by ecdysone and JH's dual interaction with USP.

1.5.4 Known roles and effects of the Juvenile Hormones

Juvenile Hormones are one of the most important insect developmental hormones, and a disruption of normal titres during this period results in death (Goodman and Cusson, 2012). Although exact levels and composition of JHs differ from insect to insect, JH levels follow a simple pattern during development: JH levels are high throughout the larval stage, then drop precipitously to permit ecdysone signalling and the onset of metamorphosis (Goodman and Cusson, 2012). Almost all research on this class of hormones has focused on this relatively narrow period of time, most likely due to the obvious commercial applications of JH disrupting compounds for pest control (as an example, the World Health Organization recommends addition of methoprene to potable drinking water as a mosquito larvicide) (Organization, 2008). Despite this focus on regulation of metamorphosis, JHs have a number of other effects that will be discussed here.

JH has long been known to affect epidermal development. Much of the work in this area has focused on the JH induced expression of cuticular proteins like LCP14/16/17 and pigmentation/melanization-related proteins like insecticyanin and dopa decarboxylase (Goodman and Cusson, 2012). Many of the LCP family of proteins (larva cuticular proteins) appear to be both up and downregulated by JH depending on the protein. It is thought that this process may be mediated by FTZ-F1 for LCP14, as the *LCP14* gene has three potential FTZ-F1 binding sites in close proximity (Goodman and Cusson, 2012). JH has effects on gene expression in other tissues besides the epidermis as well. Addition of exogenous JH to *Manduca sexta* larvae decreased expression of insecticyanin-a and insecticyanin-b in both the fat body and epidermis (Li and Riddiford, 1996).

JH is known to play a major role in gene regulation in the insect fat body (Arrese and Soulages, 2010; Goodman and Cusson, 2012). The fat body, analogous to vertebrate adipose tissue, is an organ responsible for a number of metabolic functions, including storage and release of nutrients, synthesis of proteins like vitel-

logenins used in other tissues, and even as a nutrient sensor that triggers release of insulin-like peptides in *Drosophila* (Arrese and Soulages, 2010; Rajan and Perrimon, 2012). Several research groups have demonstrated that JH controls expression of TOR signalling pathway components in *Aedes aegypti* and *Tribolium castaneum* (Shiao et al., 2008; Parthasarathy and Palli, 2011). This change in TOR signalling levels controls production of vitellogenin, a critical protein for egg laying and development (Shiao et al., 2008; Parthasarathy and Palli, 2011).

One of the most interesting effects of Juvenile Hormones is their poorly studied role in neurons and behavior. Addition of methoprene to the ant *Phiedole bicarinata* will turn worker-destined larvae into soldier ants (Wheeler and Nijhout, 1981). Higher JH levels in the last larval stage of some migratory insects could induce a stationary adult stage instead of a migratory one (Goodman and Cusson, 2012). JH is capable of affecting neuronal remodeling as well, with JH treated neurons displaying decreased dendritogenesis relative to controls (Williams and Truman, 2005). These behavioral effects appear to be caused by effects on development, as all JH treatments during these studies occured during or before metamorphosis.

Importantly, several studies have demonstrated that JH III is capable of acting directly upon neurons to affect their activity outside development. Addition of methoprene or JH III caused short-term depression of cockroach neurons both *in vitro* and *in vivo* (Richter and Gronert, 1999). JH-induced short-term depression was induced rapidly, with a 25 percent reduction in spike rate within 2 minutes, and a 75 percent reduction after 14 minutes (Richter and Gronert, 1999). Another experiment in the cricket *Acheta domesticus* indicated that JH III injection caused a translation-dependent decrease in the sound threshold required for an auditory neuron to spike by as much as 20 decibels. More importantly, this same injection of JH III could induce a change in phonotaxis (an animal's response to sound), causing animals to circle towards the side of the brain that received the injection after a sound was played via loudspeaker, with a decreased response threshold of up to 35 decibels (Stout et al., 1991). These studies make it abundantly clear that Juvenile Hormone is capable of acting directly on neurons to produce electrophysiological and behavioral changes.

Chapter 2

Methods

2.1 Insect husbandry

Unless otherwise stated, *Drosophila melanogaster* stocks used in this study were raised at 20 degrees C and 70 percent relative humidity. The stocks used for this study are as follows: Canton S, w[1118] (VDRC GD library injection strain), dcr2;$\frac{Gal80^{ts}}{CyO}$;$\frac{nysb-GAL4}{TM2}$, elav-GAL4;dcr2 (Bloomington), UAS-GFP RNAi VALIUM10 (Bloomington), UAS-Jhe RNAi VALIUM20 (Bloomington), and UAS-Jhe RNAi GD (VDRC). The identity of RNAi lines was verified by PCR before use. Stocks were raised on Nutri-Fly Bloomington Formulation pre-mixed media (Genesee Scientific). 3 to 5 day old adult flies were used for experiments, and were maintained for at least 2 days at 25 degrees C and 75 percent relative humidity beforehand. For a full list of genotypes used in figures, refer to Table 2.1.

The Liverpool strain of *Aedes aegypti* mosquitoes was used for this study. Five to seven day old adult mosquitoes were used for experiments, and maintained on 10 percent sucrose solution at 25 degrees C and 75 percent relative humidity. All insect strains were maintained on a 12 hour light / 12 hour dark circadian cycle.

2.2 Tissue sugar quantification

Tissue sugar levels for each insect were quantifies using a modified version of the methodology decribed by Miyamoto et al. (2012), where sugars are quantified

Table 2.1: Key to *Drosophila* genotypes used in figures.

Abbreviation	Genotype	Appearance
elav>Jhe RNAi	$\frac{elav-GAL4}{+}; \frac{UAS-Dcr2}{+}; \frac{+}{UAS-Jhe\ RNAi\ GD}$	Figures 3.6, 3.9, 3.10, 3.11 and 3.14
elav>+	$\frac{elav-GAL4}{+}; \frac{UAS-Dcr2}{+}$	Figures 3.6, 3.9, 3.10, 3.11 and 3.14
+>Jhe RNAi	$\frac{+}{UAS-Jhe\ RNAi\ GD}$	Figures 3.6, 3.7, 3.9, 3.10, 3.11 and 3.14
elav>Jhe RNAi #2	$\frac{elav-GAL4}{+}; \frac{UAS-Dcr2}{+}; \frac{+}{UAS-Jhe\ RNAi\ VL20}$	Figure 3.8
elav>+	$\frac{elav-GAL4}{+}; \frac{UAS-Dcr2}{+}; \frac{+}{UAS-GFP\ RNAi\ VL10}$	Figure 3.8
+>Jhe RNAi #2	$\frac{UAS-GFP\ RNAi\ VL10}{UAS-Jhe\ RNAi\ VL20}$	Figure 3.8
Dcr;Gal80^{ts};nsyb>Jhe RNAi	$\frac{UAS-Dcr}{+}; \frac{tub-Gal80^{ts}}{+}; \frac{nsyb-GAL4}{UAS-Jhe\ RNAi\ GD}$	Figure 3.7
Dcr;Gal80^{ts};nsyb>+	$\frac{UAS-Dcr}{+}; \frac{tub-Gal80^{ts}}{+}; \frac{nsyb-GAL4}{+}$	Figure 3.7

after whole animal homogenization. Although direct sugar quantification of extracted hemolymph has a number of advantages over this technique, it was deemed unfeasible over long periods of time and across large sample numbers.

In assays using *Drosophila*, ten male Canton S flies were maintained for 2 days in experimental conditions on standard fly food, and then starved on 1 percent agar upon experiment start. The equivalent experiment for *Aedes aegypti* was performed by placing 5 male mosquitoes in an empty *Drosophila* culture vial with two holes drilled in the bottom. A cotton ball moistened with dH_2O was placed at the bottom of the vial allowing mosquitoes to drink water as needed. The cotton was re-moistened every 8-12 hours by injection with additional dH_2O via pipette. For both organsims, sugar levels were quantified every 8 to 12 hours until greater than 50 percent mortality occured. Dead animals were not used for measurements. The entire experiment for both organims took place in a 25 C incubator with a 12 hour light / 12 hour dark cycle at 75 percent relative humidity.

In order to measure sugar levels, 100 µL of trehalase buffer (5 mM Tris-Hcl, 137 mM NaCl, 2.7 mM KCl, pH 6.6) was added to 5 adult male flies or 3 adult male mosquitoes for each sample. This sample was homogenized on ice and then incubated at 80C for 15 min. The resulting sample was immediately spun down at 13500g for 5 minutes and the supernatant was either frozen at -20C or used for im-

mediate sugar quantification. Glucose and trehalose quantification was performed using the Megazyme Trehalose Assay Kit (K-TREH) according to manufacturer instructions.

2.3 RNA sample preparation

For each *Drosophila melanogaster* sample, 100 males were maintained on standard fly food for two days and then placed on 1 percent agar w/v, 1 percent agar (starved) + glucose (glucose fed), 1 percent agar + yeast (yeast fed), or 1 percent agar + arabinose (arabinose fed) for 24 hours. *Aedes aegypti* samples were prepared in a similar manner, with 100 male mosquitoes per sample. Mosquitoes were fed 10 percent sucrose w/v for two days, and then either water (starved) or 10 percent sucrose solution (fed) for 48 hours. After this step, sample preparation was identical for each species.

Heads were removed and RNA was extracted after homogenization in TRIzol (Life Technologies) according to manufacturer instructions. RNA clean-up was performed using the column and protocol from Life Technologies' GeneJet RNA Purification Kit. RNA concentration and integrity were verified by spectrophotometry and agarose gel electrophoresis.

2.4 cDNA library preparation and sequencing

Before library preparation and squencing, RNA sample concentration and integrity was re-verified using a Qubit fluorimeter (Life Technologies) and BioAnalyzer (Agilent Technologies). mRNA-enriched cDNA libraries were produced using Illumina's TruSeq RNA Library Preparation Kit according to manufacturer instructions. Sequencing was either performed in a single-end 50bp configuration on either two (*Aedes aegypti*) or six (*Drosophila melanogaster*) lanes of an Illumina HiSeq 2000. Base calling, demultiplexing, and creation of FASTQ files was perfomed using CASAVA (Illumina).

2.5 RNA sequencing analysis pipeline

Reference genomes and genebuilds were obtained from either FlyBase (*Drosophila melanogaster*) or VectorBase (*Aedes aegypti*). I chose to use *Drosophila's* BDGP 5.51 (May 2013) and *Aedes's* AaegL1.3 (May 2012) annotations as those genebuilds were used to construct the OrthoDB7 orthology database used in Section 2.6 (Waterhouse et al., 2013). Bowtie2 indexes were built from reference genomes using `bowtie2-build` (version 2.1.0) (Langmead and Salzberg, 2012).

RNA-Seq reads were aligned using TopHat2 (version 2.0.10) in GTF reference-guided mode using a transcriptome index (Kim et al., 2013). Output BAM files were sorted by name-sorted (reads sorted by their aligned feature's name) with samtools and raw read counting was perfomed at the gene level using HTSeq's `htseq-count` script (version 0.6.1) under `intersectionstrict` mode (Anders et al., 2014). Differential gene expression calls were performed using the DESeq2 R package (version 1.4.5) (Love et al., 2014).

2.6 Identification of conserved transcriptomic changes

This study used OrthoDB7's *Diptera* database for determination of ortholog pairs. This means that the definition of ortholog used in this study is as identical to that used by Waterhouse et al. (2013): genes from each species pair were clustered using the best-reciprocal hits (BRHs) in an all-versus-all comparison using the SWIPE algorithm. Only the single longest transcript for each gene was considered, and minimum cutoff to be included in a cluster required an e-value below 1×10^{-3} for multiple BRHs, or below 1×10^{-6} for pair-only BRHs. A minium sequence idenity of at least 30 amino acids was also required for inclusion in a cluster (Waterhouse et al., 2013). Ortholog pairs were generated from the OrthoDB7 *Diptera* database by creating every possible pairing of *Drosophila melanogaster* and *Aedes aegypti* orthologs in each cluster using R. Significantly-regulated ortholog pairs were identified by selecting ortholog pairs where both genes in each pair had an adjusted p-value below 0.05. The significance of these genes was assessed using electronic annotations retrieved from the Ensembl database using the biomaRt R package (Durinck et al., 2005).

2.7 Validation of ortholog results

No formal statistical test exists to determine whether or not the number of observed significantly-regulated ortholog pairs is different from that which would be expected if genes were expressed randomly in each species (no conservation of genetic regulation). Nevertheless, the number of ortholog pairs expected due to random expression can be simulated. This means that the probability of the observed number of pairs being due to random expression can be calculated through iteration of this simulation in a Monte Carlo method (repeated sampling approximates the true value).

Written in Java, the simulation loads a set of orthologs from a .csv file and stores them in computer memory. A binomial distribution representing the number of significantly expressed genes in each species is then generated using a specific value of α for each species (when a gene is significantly expressed, it is defined as "on"). α represents the probability of any given gene being on in a species, and is calculated using the following formula:

$$\alpha = \frac{nSet}{setSize} \tag{2.1}$$

where:

- *nSet* = the number of elements defined by: # of significantly expressed genes in a species present in the input gene set for OrthoDB ortholog calculation for that species
- *setSize* = the number of elements in the OrthoDB input gene set

The number of differentially expressed genes for a species is determined by sampling a randomly from the binomial distribution. This number of genes is then randomly sampled from one species' genes in the ortholog set. This process is repeated for the second species using a second α calculated for that species. This give a set of genes that are "on" for each species. The simulation then compares each species' set of activated/inactivated genes, and counts the number of pairs (k) where both genes in an ortholog pair are "on". k represents the number of pairs expected due to random expression of genes for one iteration of the simulation. The

optimal number of iterations of this simulation was selected by monitoring the cumulative distribution function (CDF) and probability mass function (PMF) of k for various numbers of iterations per simulation. If the CDF and PMF remained stable across different numbers of iterations, it was assumed that the simulation had converged, approximating the true value of k. If the CDF and PMF differed between trials, more samples were required. Through this method, it was determined that an iteration depth of one million samples was sufficient to accurately determine k.

2.8 Methoprene and Precocene I feeding

Methoprene stock solution was created by the addition of 5.8 µL methoprene (5.35 µg methoprene) to 5 mL of 95 percent ethanol. In experiments where methoprene was used as a food additive, either 100 µL of methoprene stock solution or ethanol was added to 50 mL of food preparation. Oral delivery of this concentration of methoprene has previously been demonstrated as sufficient to block *Drosophila* development (Restifo and Wilson, 1998). Effectiveness of methoprene addition to food in our experiments was verified visually by delay of *Drosophila* larval development and high pupal lethality.

Precocene stock solution was prepared using 0.093 µL precocene I dissolved in 1 mL of 95 percent ethanol. 100 µL of stock solution was added per every 50 mL of food preparation. The dosage used in this study was determined by observing the sleep phenotypes of flies when subjected to a series of 2-fold dilutions of precocene beginning from 0.25 mg precocene I/mL food. The final dosage used in this study was determined by using the concentration that caused no lethality and did not affect the activity index of adult male *Drosophila* (see Section 2.12 for an overview of how this statistic was calculated). Males were used for this measurement, as preliminary experiments indicated that sex was much more sensitive to the toxic effects of precocene I. In experiments where either precocene or methoprene were fed to flies, control flies were fed an equivalent amount of ethanol as a vehicle control.

2.9 Measurement of *Drosophila* food intake

To quantify *Drosophila* food intake, we performed Capillary feeding (CAFE) assays similar to previous work (Stafford et al., 2012; Ja et al., 2007). Four male and four female flies were placed in a 15 mL conical centrifuge tube with 4 holes drilled in the lid, and allowed to recover for 30 minutes to 1 hour after anaethetization with CO_2. The lid holes were filled with the cut ends of 200 µL pipette tips and several additional holes were added near the base of the tube to allow air circulation. Each vial represents one replicate of data, and during a normal CAFE assay, up to 20 vials were fitted into holes of an airtight secondary plastic container. During each experiment, two vials did not contain flies, acting as an effective control to determine the amount of food lost to evaporation. The secondary container had a thin layer of water in the base to maintain humidity during an experiment, minimizing evaporation. Four capillaries (0.5 mM inner diameter, A-M Systems) half-filled with food solution were placed inside the pipette tip adapters at the top of each vial to act as the food source. The food solution used in this study was comprised of 50 mM D-glucose, with 0.015 percent w/v FD&C Blue No. 1 dye to aid visibility. The FD&C Blue dye was chosen because preliminary work demonstrated that it does not affect *Drosophila* food preference. After capping each capillary with a small amount of mineral oil (to limit evaporation), the level of food in each capillary was marked with a fine marker. This setup was then photographed once every hour for 20 hours. The photos were analyzed in ImageJ, and the amount of consumption was measured by calculating the distance between the level of solution in each capillary and the original food level indicated by the marker. Distances were converted from raw pixels to inches using a known reference distance on each vial, and the volume of food consumed in µL was calculated. The average amount of evaporation (as determined by the loss of food in the two controls without flies) was subtracted from test capillaries to calculate the true volume of food consumed by flies during an assay. Unless stated otherwise, all experiments were performed at 29 degrees C and 75 percent humidity with 24-hour lighting. In experiments using GAL80^{ts}, flies were maintained in these conditions for 5 days beforehand in order to fully induce GAL4 expression.

2.10 Measurement of *Drosophila* sleep

Drosophila activity and sleep was monitored using Trikinetics' DAM2 *Drosophila* Activity Monitor system. 32 flies were placed in 5 mm diameter tubes, with one end containing 1 cm worth of food. The food used for these experiments either consisted of 2 percent agar plus 5 percent sucrose w/v (sucrose food) or 2 percent agar (agar food). When methoprene was used as a food additive, either methoprene solution or ethanol was added directly to the food in the activity monitor tubes as described above. Experiments were run at 25 degrees C and 75 percent humidity on a 12 hour light / 12 hour dark cycle. Flies were placed inside the activity monitor tubes for at least two days before each experiment to acclimate to experimental conditions and the DAM2 system. Sleep suppression was assessed by placing the flies on sucrose food for 24 hours, followed by agar food for 24 hours, and finally another period of 24 hours of sucrose food. *Drosophila* activity was recorded every 5 minutes. Sleep and activity phenotypes were assessed using the `actmon` R package written for this study. Data from flies that died during the course of an experiment were discarded.

2.11 Measurement of *Drosophila* starvation sensitivity

Drosophila starvation sensitivity was assessed using Trikinetics' DAM2 system. Briefly, 32 flies were placed in 5 mM diameter tubes containing 1 cm worth of agar food in one end. The activity of the flies was recorded every five minutes until all flies had succumbed to starvation. Experiments were run at 25 degrees C and 75 percent humidity on a 12 hour light / 12 hour dark cycle. Starvation sensitivity and survival was assessed using the `actmon` R package.

2.12 `actmon` R package

To analyze data produced by the DAM2 system, I developed the `actmon` R package to effectively quantify *Drosophila* sleep and activity behavior. `actmon` allows fast, easy, and reproduceable analysis of data produced by Trikinetics' devices in R. A S4 helper class is provided to hold an activity monitor experiment and its metadata, along with a number of methods to simplify data handling and analy-

sis. The package reduces complex tasks like removing dead animals or syncing an experiment's output data to the light/dark cycle in an incubator to simple one-line functions. All functions and methods are designed to maintain the original Trikinetics' format and experimental metadata at every step, allowing `actmon` to be used either by itself or in conjunction with other sleep analysis tools like PySolo, ActogramJ, or FaasX (Gilestro and Cirelli, 2009; Schmid et al., 2011). `actmon` also provides methods to convert an experiment to the widely-used tidyR "long-data" format, enabling easy follow-up analysis in R. `actmon` also provides wrapper functions that provide the ability to produce publication-ready plots using the `ggplot2` graphics package.

Sleep was defined as 5 minutes of no activity, in accordance with the accepted definition established by Hendricks et al. (2000). Time of death was determined by identifying the final contiguous period of time in which no movement occurred for the rest of the experiment ($<$5 counts / hour). As an additional requirement, the duration of this final motionless period must be at least 4 hours in length before it can be considered death. Activity index (locomotor activity normalized to time awake) was calculated by the following formula: # of activity counts per day / # of minutes awake per day (Kume et al., 2005). Sleep bouts were detected by identifying contiguous periods of sleep after sleep detection has been performed. The source code and installation instructions for this package are publicly available online at: https://github.com/kazi11/actmon.

2.13 Plotting and statistics

With the exception of the ortholog statistical simulation described in Section 2.7, all statistical calculations in this study were performed using base R (version 3.1.0). All plots were produced in either R or Microsoft Excel.

Chapter 3

Results

3.1 Characterization of insect hunger

This study relied upon sequencing the mRNA of *Drosophila melanogaster* and *Aedes aegypti* in a food-deprived state. In order to select timepoints in each species that represented a similar level of starvation, the levels of sugar present in the insects' whole-body tissue were quantified. This allowed identification of a time point in which the starvation level of each insect species was roughly equivalent.

Glucose and trehalose are the primary form of stored energy in the hemolymph of most insects, and offer an excellent snapshot of starvation state in *D. melanogaster* and *A. aegypti* (Wyatt, 1961). Lending support to this approach, it has been previously demonstrated that internal sugar levels are highly responsive to nutritional state (Wyatt, 1961).

3.1.1 Determination of *Drosophila* starvation state

Starvation has been relatively well characterized in *Drosophila melanogaster*. If levels of glucose and trehalose in *Drosophila* had noticeably decreased by the time point the literature defines as starvation (24 hours), it would support the conclusion that measurement of sugar levels was an accurate and effective way of characterizing hunger (Fujikawa et al., 2009; Farhadian et al., 2012). This is especially important given that the starvation sensitivity of *Aedes aegypti* males is relatively poorly understood.

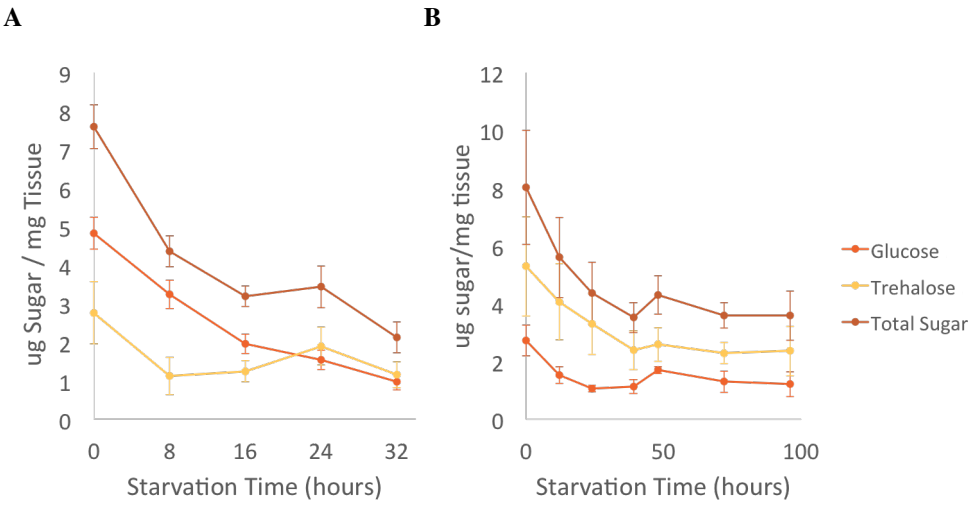

Figure 3.1: Quantification of insect sugar levels.
(A) Glucose, trehalose, and total sugar levels of male Canton S *D. melanogaster* during starvation (n = 8 replicates of 10 flies for each timepoint, 5 flies used for sugar measurement). **(B)** Sugar levels of male *A. aegypti* during starvation (n = 4 replicates of 5 mosquitoes for each timepoint, 3 mosquitoes used per measurement). Error bars for both subfigures represent the standard error of the mean.

Adult *Drosophila melanogaster* males were placed in culture vials filled with 1 percent agar and a subset of experimental animals were subjected to sugar quantification every 8 hours until significant mortality occurred. We chose to use only male flies for this experiment due to the fact that sequencing was to be performed on only male flies and male mosquitoes. Female *Aedes aegypti* mosquitoes feed on blood as adults, and it seemed likely that this additional feeding modality would complicate an inter-species comparison with *Drosophila*.

The results of sugar quantification during starvation in *Drosophila* are presented in Figure 3.1A. Total sugar levels drop following starvation, decaying in a rapid manner until plateauing at the 24 hour timepoint. The levels of glucose and trehalose declined at similar rates. This 24h timepoint was used for sample preparation in the subsequent sequencing experiments. It is important to note that 24 hours without food is a widely-accepted definition of starvation used throughout the field of *Drosophila* gustation, and was selected for several similar studies

examining transcriptional changes induced by starvation (Fujikawa et al., 2009; Farhadian et al., 2012). The fact that our definition of starvation based on sugar measurements so closely matched literature values affirmed that this methodology would give an accurate estimate of "hunger" in *A. aegypti*.

3.1.2 Determination of *Aedes* starvation state

Starvation state for adult male *A. aegypti* was assessed using the same methodology used for *Drosophila* (see Figure 3.1B). Tissue glucose and trehalose levels decreased in an identical manner to that observed in flies, rapidly decaying upon starvation. Interestingly, *A. aegypti* are almost twice as resistant to starvation compared to *Drosophila*. Whereas almost all male flies had died within 48 hours without food, a number of male mosquitoes were still alive after 96 hours without food. Nevertheless, sugar levels in *A. aegypti* plateaued around 48 hours of starvation. The 48 hour timepoint was not chosen arbitrarily- total sugar levels of each insect were roughly equal to that observed in *Drosophila* at 24 hours (4 µg per mg tissue). This 48 hour timepoint was defined as the "starved" state used for RNA sequencing.

3.2 RNA sequencing reveals conserved transcriptomic changes

After determining what constituted a starved state for *D. melanogaster* and *A. aegypti*, I sought to identify the transcriptomic changes that occurred in each animal. These insects were either fed or starved (24h for *D. melanogaster*, 48h for *A. aegypti*) in an identical manner to that described in Section 3.1. Several additional "fed" samples of *Drosophila* were also prepared in addition to glucose food (nutritive sugar), in which flies were fed on various food sources, including yeast (contains significant amino acids and fat), and arabinose (a non nutritive sugar). The heads of each animal were removed at the timepoints of interest and mRNA was prepared for sequencing at these timepoints. We chose to sequence insect heads (as opposed to the whole animal), as this is an effective method to enrich for genetic changes specific to neuronal tissues. We chose to focus on studying neuronal tissues as these are the cells responsible for directly controlling behavior.

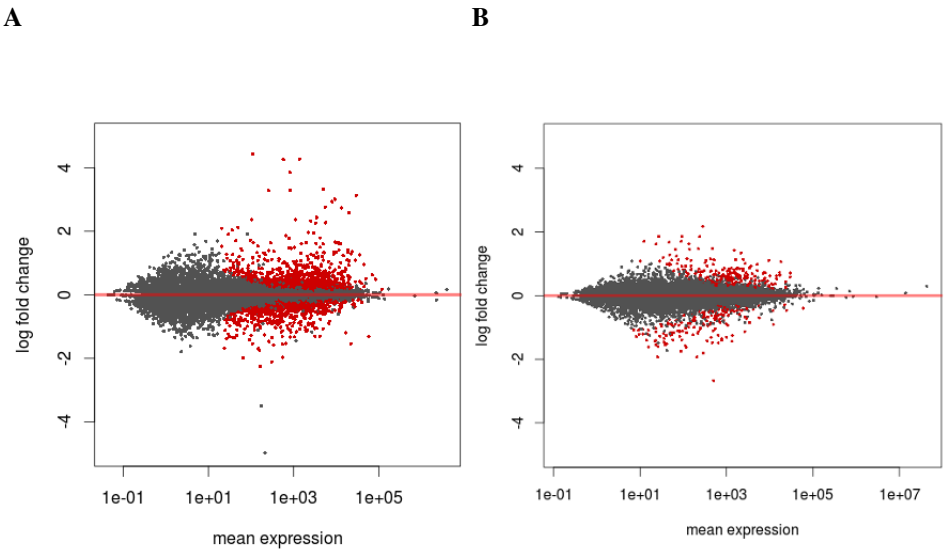

Figure 3.2: Overview of RNA sequencing results.
MA plots for *Drosophila melanogaster* **(A)** and *Aedes aegypti* **(B)**. The data for *Drosophila* shown here represent a comparison between starved and glucose-fed flies, whereas data in **B** are a comparison of starved and sucrose-fed mosquitoes. Each point represents the expression of a single gene. Red points are significantly regulated, with an adjusted p-value < 0.05. Mean expression is in terms of raw read counts normalized to library size, and change in expression is in terms of $\log_2(starved/fed)$. Note that $\log_2$ fold-change estimates for genes with low read count or high dispersion have been shrunk using the DESeq2 R package.

An overview of these sequencing results is presented in Figure 3.2.

1519 genes were significantly regulated in *D. melanogaster* by starvation, with 891 genes upregulated and 621 genes downregulated in starved agar-fed samples relative to glucose-fed controls. 307 genes were significantly regulated in *A. aegypti* by starvation, with 181 genes upregulated and 126 downregulated in starved water-fed samples relative to sucrose-fed controls. This ran contrary to my prediction that the primary response in both organisms would be to downregulate genes and cellular pathways in an effort to limit protein biosynthesis and metabolism.

3.2.1 Identification of conserved transcriptomic changes

The next step of our analysis sought to examine the conserved transcriptomic changes occuring in these organisms. In order to accomplish this, we would need to develop our own novel method of analysis, raising a number of important questions. How can transcriptomic changes in one organism be compared to that of another? The genomes of *D. melanogaster* and *A. aegypti* are not a one-to-one match. A method would need to be developed to match gene expression changes in one species with that of another. Furthermore, once such a method had been developed, I would need to establish a method of statistically validating this approach.

In order to identify genes that showed conserved regulation across both species, I established the following criteria:

1. There must be a biologically meaningful means of matching a gene in one species with that of another.

2. For the genetic changes of a matched pair to be considered "significant," the change in expression for each gene in the pair must be significant, with an adjusted p-value < 0.05.

3. The number of significantly regulated matched gene-pairs must be greater than that would be expected due to random upregulation/downregulation of unrelated sets of genes.

4. There should be some amount of correlation between gene expression changes in each organism.

To address item 1 of this definition, we chose to use the biological concept of orthology as a means of matching genes in one organism with those of another. Orthologs are sets of genes in different species that diverged from a common ancestor gene through the process of speciation. As a result, orthologs typically share significant sequence identity and often share common molecular functions as well. Although this is a useful definition that satisfies the requirement of a "biologically meaningful" method of matching genes in one species with those of another, it

is not "computationally meaningful." To elaborate, it would be impossible to apply the biological definition of orthology on a genome-wide scale across multiple species because it does not supply strict criteria that can be evaluated by a computer. Because of this limitation, it was necessary to use a computational definition of orthology instead. We chose to use the definition specified by Waterhouse et al. (2013), using information provided by the OrthoDB7 orthology database. Due to the importance of definitions in this context, I will outline the procedure Waterhouse et al. used to calculate orthologs here: the protein sequence of the longest transcript for each gene in a species was compared against those of every other species in the OrthoDB7 database using the SWIPE algorithm. If two sequences shared significant sequence identity, they were declared as orthologs and included in a cluster of other matches (Waterhouse et al., 2013). We created the set of orthologous gene-pairs used in this study by creating every possible pairing of *D. melanogaster* and *A. aegypti* genes from these clusters. This means that the set of orthologs used in this study is identical to those defined by OrthoDB7. A more detailed explanation of this procedure can be found in Section 2.6.

After obtaining the list of ortholog pairs for both species, we sought to find which ortholog pairs were significantly regulated. I declared any pair in which the adjusted p-values for genes from both species were below 0.05 as significant. Once this operation had been performed, I was left with 117 significantly regulated pairs. To feel confident in using these data, I needed to determine if the significantly regulated orthologs indicative of any biological trend, or if this was a result caused by significant genes from both species simply being regulated in the same direction by chance.

3.2.2 Validation of ortholog results

I wanted to understand if the observed number of significantly ortholog pairs differed from what would be expected if gene regulation in each species were random (note that this is a separate measurement from the probability of a given gene being differentially expressed calculated by DESeq2). Unfortunately, there are no formal statistical tests capable of answering this question. The nature of orthology means that a gene in one species may have any number of equivalents in another species,

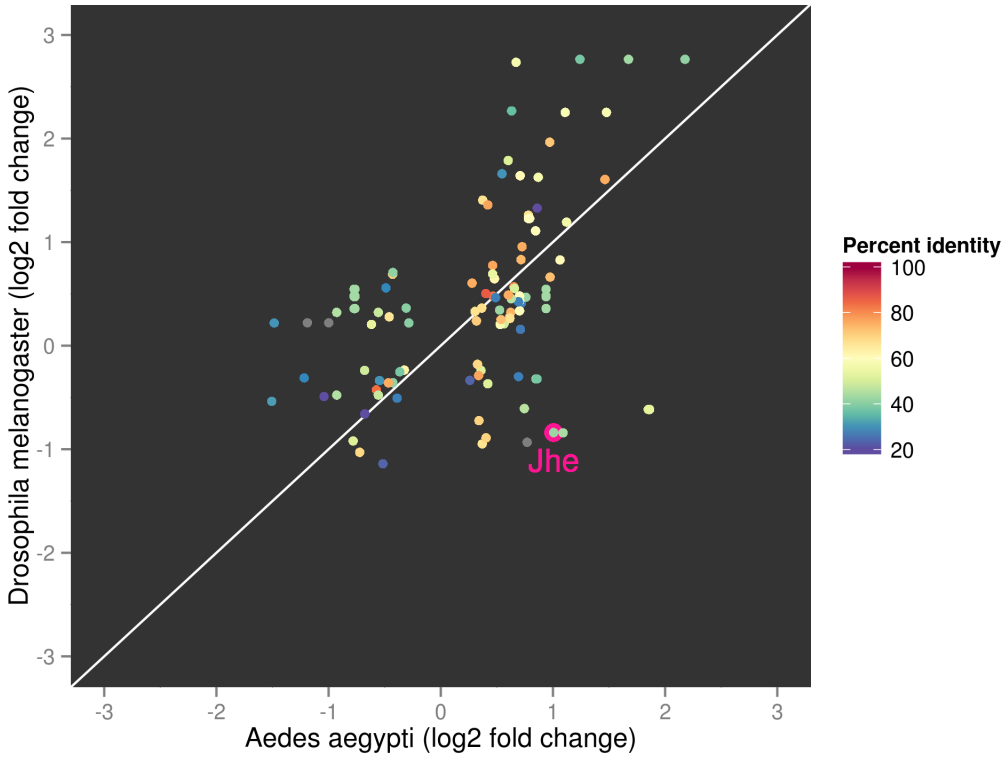

Figure 3.3: Orthologous gene-pairs significantly regulated by starvation. This plot contains all of the significantly regulated ortholog pairs where the adjusted p-value for each gene in a pair's change in expression was below 0.05. Percent identity was calculated as the percent sequence identity between the *Drosophila melanogaster* gene and its *Aedes aegypti* equivalent. The pair comprising *Jhe* and its ortholog, *AAEL005178* has been highlighted in magenta. The white line in this figure represents a one-to-one correlation in gene expression between orthologs in a pair.

including none, complicating any potential analysis. However, the result expected if our null hypothesis were true (the observed number of orthologs is equal to the number expected if gene regulation was random) is specific enough to be simulated with a computer. Although a simulation done in this manner may differ from run to run, enough iterations of the program will eventually approach the true value of the simulated statistic. This approach to a statistical problem is referred to as a Monte Carlo method.

Our simulation capitalized on the fact that random genetic regulation in both species could be calculated easily. A probability of any given gene being regulated in a species was computed by taking the number of genes significantly regulated in the ortholog set divided by the total number of genes for that species in the ortholog set. This probability α, was used to generate a binomial distribution of the number of significantly regulated genes in a given simulation run. During each iteration of the simulation, we selected a random set of X number of genes (X was determined by randomly sampling from that species' binomial distribution) from each species as "on", or significantly regulated. The list of genes "on" in each species was then compared to that of the other species and the number of significantly regulated orthologs (defined as k) was determined by counting the number of matches where both genes in an orthologous pair were "on." For more details on this simulation, see Section 2.7.

Using the methodology described above, we calculated the values of α for *A. aegypti* and *D. melanogaster* as 0.0198 and 0.1425, respectively. Running one million iterations of the simulation resulted in a mean k of 37.65. Remarkably, the highest observed value of k observed was 83. This indicates that the probability of observing 117 orthologous pairs due to random genetic regulation is literally less than one in a million. The full results of the simulation are summarized in Figure 3.4.

Although the number of ortholog pairs was significantly greater than what would be expected, testing for correlation in gene expression in *A. aegypti* and *D. melanogaster* would add an additional level of validation to our hypothesis that the gene expression in the two species is conserved. Gene expression among the significantly-regulated orthologous pairs was positively correlated with a Pearson's ρ product-moment correlation coefficient of 0.40 ($p < 0.001$). Taken together with our simulation results, the data demonstrate that many changes in gene expression induced by hunger are conserved between *A. aegypti* and *D. melanogaster*.

3.2.3 *Jhe* is regulated significantly by hunger

I examined the list of conserved ortholog pairs between *A. aegypti* and *D. melanogaster* for candidate genes likely to be capable of controlling behavior, with a focus on

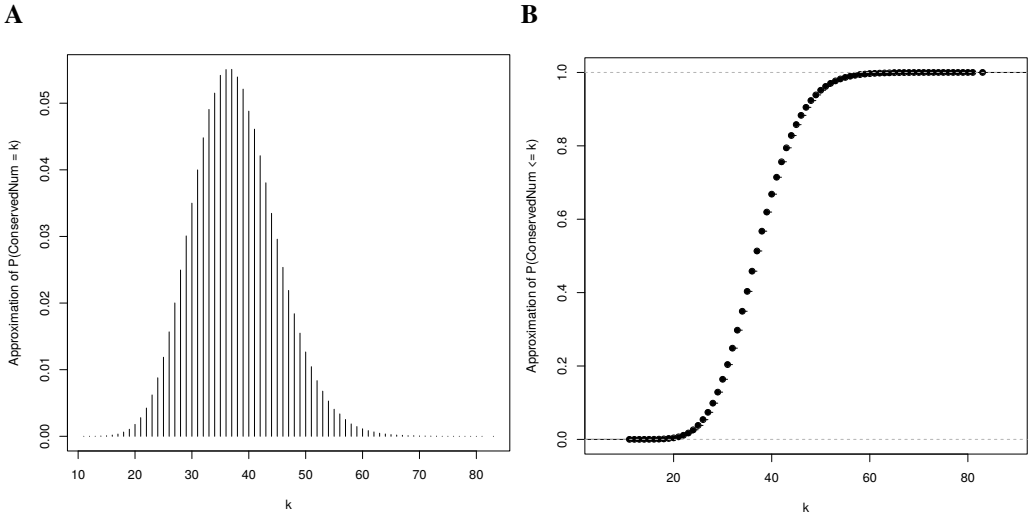

Figure 3.4: Ortholog simulation results.
(A) Probability mass function of the orthologous pair statistical simulation described in Section 2.7, using α values of 0.0198 and 0.1425 for *A. aegypti* and *D. melanogaster* across one million iterations. **(B)** Cumulative distribution function of the same simulation results from **A**.

anything capable of potentially affecting neuronal activity. Interestingly, Juvenile Hormone esterase (*Jhe*) was significantly downregulated in flies, with a $\log_2$ fold-change of -0.84. One of *Jhe*'s *Aedes aegypti* orthologs, *AAEL005178*, was significantly regulated as well, with a $\log_2$ fold-change of 1.01. Although these genes are regulated in opposite directions in each insect, *Jhe* is known to be involved in hormone metabolism, and has been shown to degrade the insect hormone Juvenile Hormone (JH) *in vitro* (Crone et al., 2007). Bioinformatic analyses indicates that AAEL005178 is a type-B carboxylesterase/lipase, a shared feature with *Jhe*. Additionally, *AAEL005178* and *Jhe* cluster together in OrthoDB7 ortholog group EOG7T22TR with other annotated Juvenile Hormone esterases from the mosquitoes *Anopheles gambiae* and *Culex quinquefasciatus* (Waterhouse et al., 2013). Given this information, it seems likely that *AAEL005178* also encodes a Juvenile Hormone esterase.

In addition to my analysis of the conserved changes between *A. aegypti* and *D. melanogaster*, I chose to examine the genetic changes caused by multiple forms

of nutrient deprivation in *Drosophila* (Figure 3.5). Intriguingly, two JH-related genes were downregulated in the comparison between both the glucose/agar-fed and yeast/agar-fed conditions: *Jhe* and Takeout (*To*). Takeout is currently thought to be a Juvenile Hormone binding protein, protecting JH from degradation as it circulates throughout an insect's hemolymph (Sarov-blat et al., 2000; Meunier et al., 2007). Taken together with the sequencing data from *A. aegypti*, it seemed likely that Juvenile Hormone esterases and Juvenile Hormone played some kind of role in the response to starvation.

3.3 *Jhe* is necessary for proper feeding behavior

Although our RNA-Seq data identified Juvenile Hormone signaling as of potential interest, it did not establish whether or not the pathway had any effect on *D. melanogaster* or *A. aegypti* adults. Juvenile Hormone is primarily thought to be an insect developmental hormone. Does it affect adults as well? To answer this question, I chose to study *Jhe* using *D. melanogaster* as a model. As I was interested in understanding if *Jhe* played a role as a regulator of behavior, I decided to knock down expression of the gene using a pan-neuronal RNAi approach. Since *Jhe* was downregulated by starvation, we postulated that the gene may have a role in feeding behavior. *Jhe* RNAi flies and controls were assessed for food consumption phenotypes using the CAFE assay.

Interestingly, pan-neuronal *Jhe* RNAi flies consumed significantly more food than controls when they were fed 50 mM glucose (Figure 3.6). This phenotype was replicated regardless of what sugars the flies were presented with. It is important to note that this occurred regardless of a sugar's nutritional value, as *Jhe* RNAi flies even consumed more than controls when presented with the non-nutritive sugars L-fucose and arabinose. This indicates that *Jhe* does not affect caloric sensing of food- it affects consumption of all sugars equally. Additionally, *Jhe* RNAi flies still ate more food when presented with mannose, which is a relatively unpalatable nutritive sugar (it is typically necessary to combine mannose with a more palatable sugar like sucrose before flies will eat it readily). Taken together, these results seem to suggest that *Jhe* knockdown triggers a starvation-like state in *Drosophila*.

Given that JH serves as an important regulator of development, one poten-

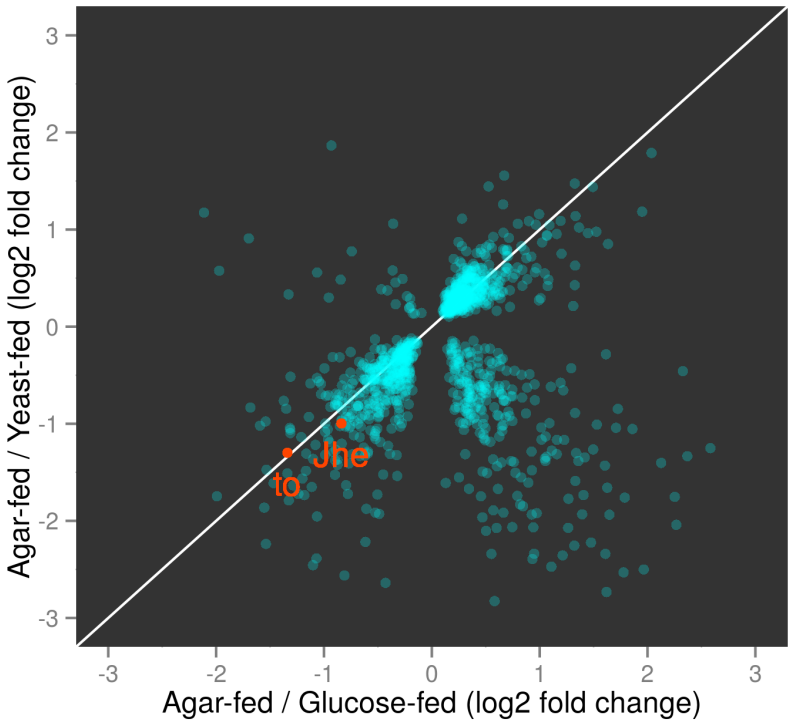

Figure 3.5: JH-related genes are downregulated in multiple forms of nutrient deprivation in *Drosophila*.
Significantly-regulated genes between glucose and agar feeding (x-axis) have been plotted against those genes regulated between yeast and agar feeding (y-axis). To be included on this plot, genes must have been significantly regulated in both conditions, with an adjusted p-value less than 0.05. The white line in this figure represents a one-to-one correlation in a gene's expression in each condition. *Juvenile hormone esterase (Jhe)* and *Takeout (to)*, two genes implicated in Juvenile Hormone signalling, have been highlighted in orange to aid visibility.

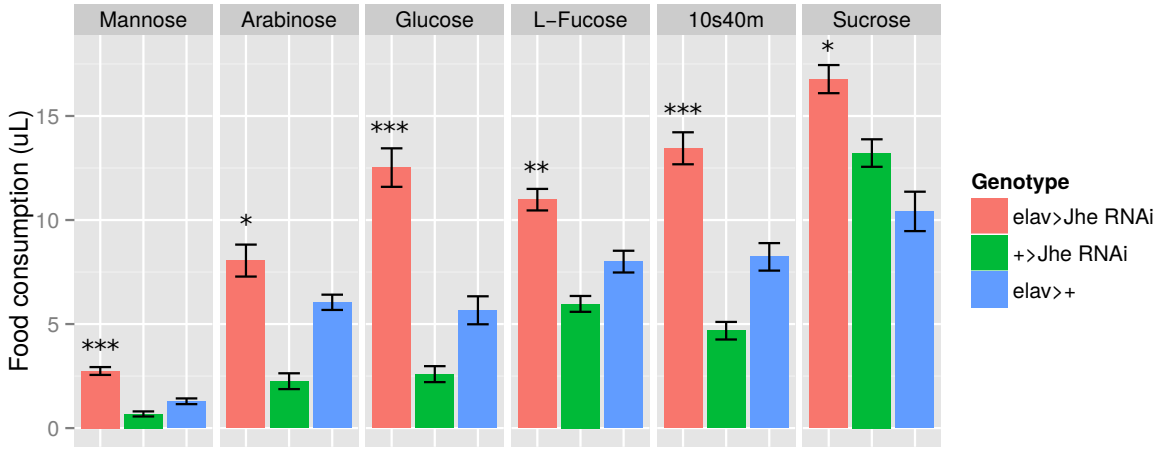

Figure 3.6: *Jhe* is a modulator of feeding behavior.
Jhe RNAi flies' consumption was characterized using the CAFE assay on a variety of food sources. Neuronal *Jhe* RNAi flies ate significantly more than controls regardless of which sugar they were fed. All sugars tested were at a concentration of 50 mM. 10s40m comprises a mixture of 10 mM sucrose and 40 mM mannose. Error bars represent standard error of the mean. n = 5-7 replicates of 8 flies per genotype for each sugar. Asterisks indicate a significant difference from controls as measured by One-Way ANOVA followed by post-hoc Tukey HSD test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

tial concern is that the observed effect may be of developmental origin. To exclude this possibility, we performed another experiment, where *Jhe* knockdown was restricted to *Drosophila's* adult stage using the temperature-sensitive isoform of the GAL4-repressor GAL80^{ts}. Importantly, when *Jhe* knockdown was restricted to adults, the feeding phenotype was reproduced (Figure 3.7). Moreover, when GAL4-mediated RNAi was turned off at room temperature, there was no difference between the RNAi flies and their isogenic controls, indicating that this approach was effective in restricting RNAi expression to the adult stage (Figure 3.7). These results indicate that *Jhe's* feeding phenotype is not due to a developmental defect. It is also important to note that this experiment utilized a different neuronal GAL4 driver, nysb. The fact that the same phenotype was reproduced using two separate neuronal drivers indicates that *Jhe* is indeed expressed in neurons, and the

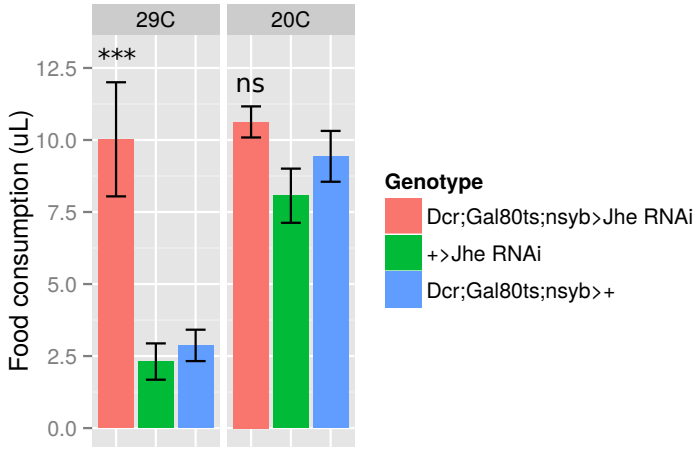

Figure 3.7: *Jhe's* phenotype is not a developmental defect.
Food consumption of *Jhe* RNAi flies when knockdown was induced in adults at 29C, or was never induced at room temperature (20C). Flies in both experiments were presented with 50 mM glucose as a food source. Error bars represent the standard error of the mean. Consumption was measured at either 12 hours after experiment start (29C) or 30 hours after experiment start (20C). Error bars represent standard error of the mean. n = 5-6 replicates of 8 flies per genotype/condition. Asterisks indicate a significant difference from controls as measured by Two-Way ANOVA followed by post-hoc Tukey HSD test (*** = $p < 0.001$, ns = not significant).

phenotype is not an artifact of "leaky" GAL4 expression in other, non-neuronal tissues.

Finally, it was critical to exclude the possibility that the phenotype arising from *Jhe* knockdown were a result of RNAi off-target effects. To do this, pan-neuronal knockdown of *Jhe* expression was performed with a new RNAi line. The second RNAi line targeted a separate region of the *Jhe* transcript relative to the RNAi line used in Figure 3.6. This resulted in an identical phenotype to that demonstrated earlier: increased consumption of food (Figure 3.8). From the data shown here, it seems clear that *Jhe* may function as a novel regulator of hunger-induced feeding behavior in insects. RNAi knockdown of Jhe is consistent with the downregulation that this gene normally undergoes during starvation in *Drosophila* (see Figure 3.5).

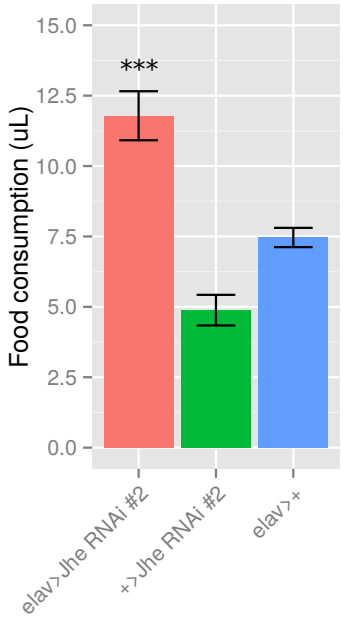

Figure 3.8: *Jhe's* phenotype is not an off-target effect.
Food consumption of *Jhe* RNAi #2 flies and controls (GAL4/+ and RNAi/+) quantified by CAFE assay. Flies were fed 50 mM glucose for 12 hours, after which their consumption was measured. Error bars represent standard error of the mean. n = 9-13 replicates of 8 flies each. *** = $p < 0.001$ by One-Way ANOVA with post-hoc Tukey HSD.

3.4 *Jhe* knockdown increases starvation-induced sleep suppression

Increased feeding is not the only behavioral change an insect will make upon starvation. The starvation response in insects includes a wide-range of behaviors, including starvation-induced sleep suppression. Sleep suppression is a relatively well-characterized phenotype where hungry *Drosophila* will avoid sleeping in order to forage for food (Keene et al., 2010). The test to examine this phenotype is simple: adult *Drosophila* are placed on food for 24 hours, starved for 24 hours, and then allowed to recover with food for a final 24 hours. During the 24 hours in which flies are deprived of food, they will suppress sleep. The amount of locomotor ac-

tivity and sleep can be quantified using Trikinetics' *Drosophila* Activity Monitor and analyzed in my `actmon` R package (for details of how sleep calculations are performed with `actmon`, see Section 2.12.

When *Jhe* knockdown flies were assessed for sleep suppression, they demonstrated increased activity relative to controls during the starvation period (Figure 3.9A). This increased activity resulted in knockdown flies sleeping significantly less than controls (Figure 3.10). This sleep suppression effect was identical to that described by Keene et al. (2010), where this sleep suppression only occurred during the second 12 hour period of starvation. This effect has previously been shown to occur during this second 12 hour period, irrespective of light or dark status (Keene et al., 2010).

One question this phenotype raised was whether this effects on sleep could simply be attributed to increased locomotor activity. This was assessed by examining the activity index of flies during the assay, a measure of activity counts normalized to the amount of time an animal spends awake. Hyperactive flies will have a higher activity index than controls, and hypoactive flies with a lower activity index may indicate some form of locomotor defect. *Jhe* RNAi flies showed an identical activity index to controls, indicating that locomotor activity was unaffected by the knockdown (Figure 3.9B). *Jhe's* activity phenotype is therefor likely a direct result of those flies sleeping less than controls. This held true regardless of whether or not males or females were tested, indicating that this is not a sex-specific phenotype. Taken together, these results indicate that *Jhe* knockdown increases starvation-induced sleep suppression. This supports the hypothesis that *Jhe* regulation is a driver of starvation-induced behavior. Neuronal knockdown of *Jhe* causes flies to both increase feeding and suppress sleep, indicating that downregulation of this gene during starvation may be what causes these phenotypes in wild-type flies.

3.5 *Jhe* knockdown does not increase starvation sensitivity

Neuronal *Jhe* downregulation results in a starvation-like behavioral state, increasing both feeding and starvation-induced sleep suppression. The most obvious ex-

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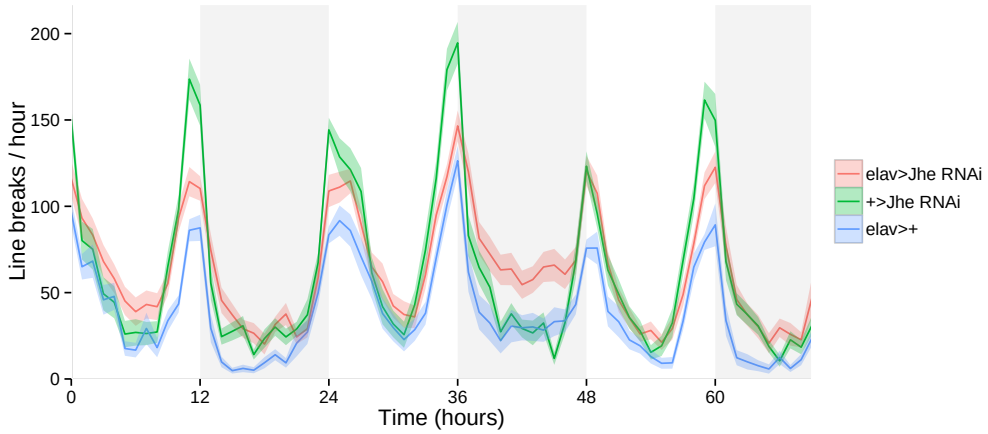

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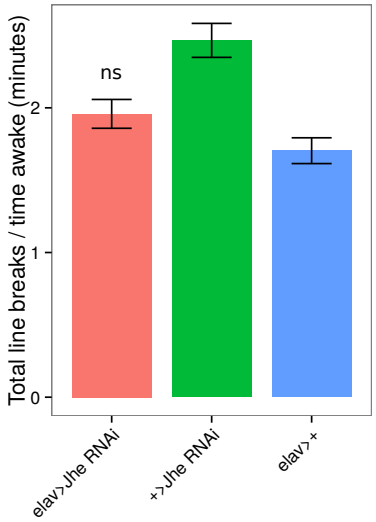

Figure 3.9: *Jhe* knockdown alters *Drosophila* activity.
(A) A measurement of raw *Drosophila* activity, as measured in total line breaks/hour. Flies were deprived of food between hours 24 and 48. Light or dark regions represent whether experimental lighting was on or off. **(B)** Average activity index (total activity normalized to time awake) for each genotype for the time period between 36 and 48 hours from the plot in **A**. Error bars in **B** and colored regions in **A** represent the standard error of the mean for each data point. n = 30 - 36 flies per genotype. ns = not signifcant by One-Way ANOVA followed by post-hoc Tukey HSD test.

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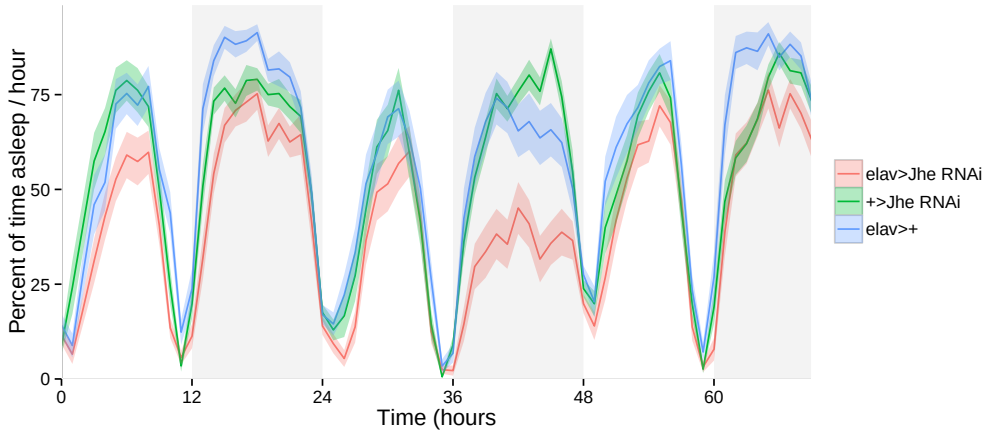

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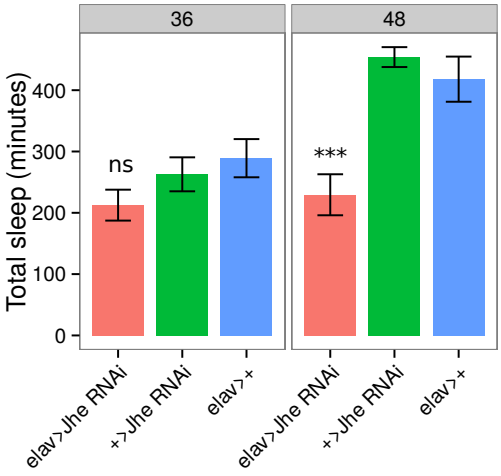

Figure 3.10: *Jhe* knockdown increases starvation-induced sleep suppression. **(A)** A measurement of *Drosophila* sleep, in percent of time asleep per hour. Flies were deprived of food between hours 24 and 48. Light or dark regions represent whether experimental lighting was on or off. **(B)** Total sleep for each genotype for the time period between either 24 and 36 hours **(36)**, or 36 and 48 hours **(48)** from the plot in **A**. Error bars in **B** and colored regions in **A** represent the standard error of the mean for each data point. n = 30 - 36 flies per genotype. *** indicates significance versus controls with $p < 0.001$ by One-Way ANOVA followed by post-hoc Tukey HSD test, ns = not significant.

planation for these phenotypes is that *Jhe* downregulation may actually cause starvation itself. If this were the case, and *Jhe* knockdown causes flies to become energetically starved (instead of just acting hungry), *Jhe* RNAi flies would have increased starvation sensitivity relative to controls, dying quickly when deprived of nutrients. To test this hypothesis, starvation sensitivity was assessed using the *Drosophila* Activity Monitor. RNAi flies and controls were placed on agar food and survival time was assessed using the `actmon` R package. The starvation sensitivity of male and female flies was quantified separately, as the sexes differ dramatically in both body size and starvation tolerance.

Jhe RNAi flies did not exhibit differential starvation sensitivity relative to controls, indicating that neuronal downregulation of this gene does not affect an animal's energetic state (Figure 3.11). This held true for both male and female *Drosophila*, again indicating that *Jhe's* effects are not sex-specific. The only remaining explanation for *Jhe's* phenotypes was that this gene was directly acting to control behavior, instead of indirectly causing a behavioral change by starving an animal.

3.6 Methoprene feeding results in a *Jhe*-like phenotype

From the evidence discussed so far, it is clear that *Jhe* knockdown during starvation acts to induce hunger-related behaviors. However, the mechanism through which *Jhe* induces these behavioral changes remained unknown. To tackle this question, we chose to study the most obvious candidate: the insect hormone Juvenile Hormone III. *Juvenile Hormone esterase* has been previously shown to degrade a wide number of JH derivative compounds *in vitro*, and it has been postulated that *Jhe* is the only esterase likely to degrade this hormone *in vivo* (Campbell et al., 1998; Crone et al., 2007). Because of this evidence, it seemed likely that *Jhe* exerts its behavioral effects by degrading JH. According to this hypothesis, downregulation of *Jhe* during starvation would result in increased JH levels, which would trigger behavioral changes through activation of the MET-GCE heterodimeric receptor or USP. To test this, we would need some way of artificially increasing flies' JH levels.

As mentioned earlier in Section 1.5.1, the JH synthesis pathway in *Drosophila* is poorly understood. As a result, we chose to artificially increase JH signaling

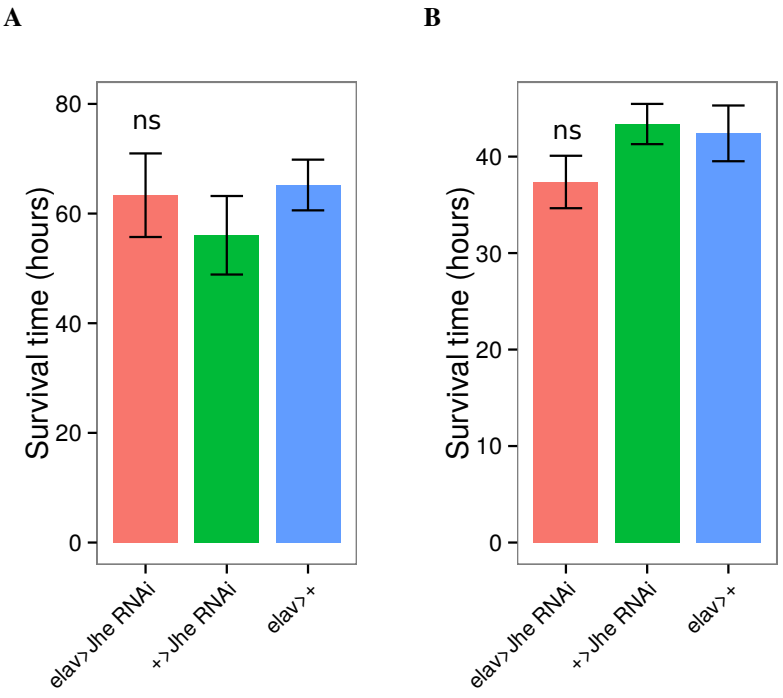

Figure 3.11: *Jhe* knockdown does not affect starvation sensitivity.
Female **(A)** and male **(B)** *Drosophila* starvation sensitivity was assessed by measuring survival time of flies during absolute starvation. Survival time was measured in hours. No significant difference was observed between knockdown flies and controls. Error bars represent the standard error of the mean for each data point. n = 32 flies per condition. ns = not signifcant by One-Way ANOVA followed by post-hoc Tukey HSD test.

through the use of methoprene. Methoprene is a synthetic analog of Juvenile Hormone that binds to JH receptors with high affinity and specificity and cannot be degraded by insect Juvenile Hormone esterases and Juvenile Hormone epoxide hydrolases. This chemical is effective when ingested orally, and is commonly used as an insecticide due to its activation of the JH signaling pathway (Organization, 2008). Because of these factors, feeding flies methoprene seemed like a perfect mechanism of simulating *Jhe* downregulation by increasing JH signaling.

Adult *Drosophila* were fed standard cornmeal food containing either metho-

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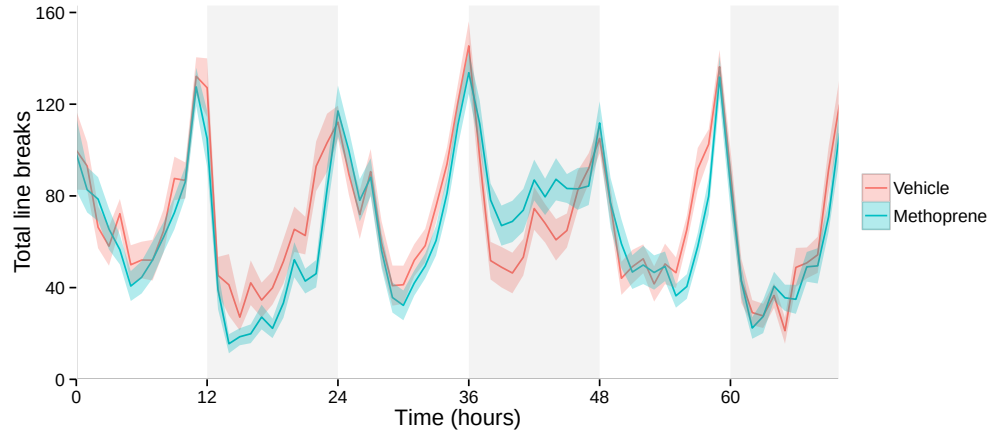

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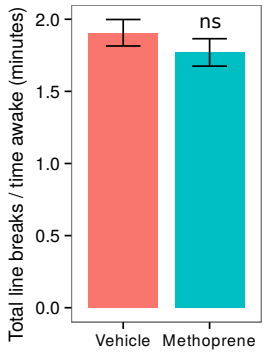

Figure 3.12: Methoprene alters *Drosophila* activity in a *Jhe*-like manner.
(A) A measurement of raw *Drosophila* activity when fed methoprene, as measured in total line breaks/hour. Flies were deprived of food between hours 24 and 48. Light or dark regions represent whether experimental lighting was on or off. **(B)** Average activity index (total activity normalized to time awake) for each genotype for the time period between 36 and 48 hours from the plot in **A**. Error bars in **B** and colored regions in **A** represent the standard error of the mean for each data point. n = 32 flies per condition. ns = not signifcant by One-Way ANOVA followed by post-hoc Tukey HSD test.

prene or ethanol (the vehicle used to dissolve methoprene) for two days before measuring their behavior. Methoprene/vehicle feeding was continued during these experiments. Methoprene feeding increased starvation-induced sleep sensitivity in a *Jhe*-like manner. Methoprene-fed flies slept significantly less than vehicle-fed controls when starved (Figure 3.13). Although methoprene increased total activity of flies during the same period, the activity index of flies was unaffected (Figure 3.12). As before, the phenotype was identical to that described by Keene et al. (2010), where sleep suppression occurred during the second 12 hours of the starvation period. This is identical to the phenotype observed during *Jhe* knockdown- an increase in starvation induced sleep suppression without affecting the total amount of activity when animals were awake.

3.7 Precocene I rescues *Jhe* knockdown

The results discussed in Section 3.6 indicated that increasing JH titers reproduced the effects of *Jhe* knockdown. By the same reasoning, if JHE exerted its behavioral effects by degrading JHs, then artificially decreasing JH titers should "rescue" the phenotype produced by *Jhe* knockdown. To test this possibility, we fed *Jhe* knockdown flies the anti-juvenoid agent precocene I. Precocene I is a relatively well-characterized drug capable of blocking JH synthesis. This drug acts upon the corpora allata directly, decreasing its secretory activity (Wilson et al., 1983).

As demonstrated in Figure 3.14, precocene I feeding rescues the effects of *Jhe* knockdown without affecting normal waking activity. Precocene I was specifically able to rescue the increase in sleep suppression of *Jhe* knockdown flies. This phenomenon was not sex-specific, and further strengthens the conclusion that JHE exerts its effects through JH.

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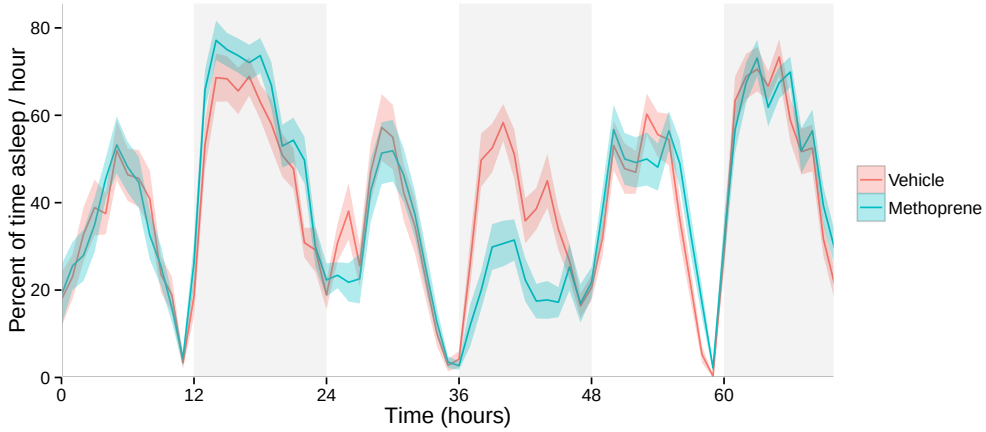

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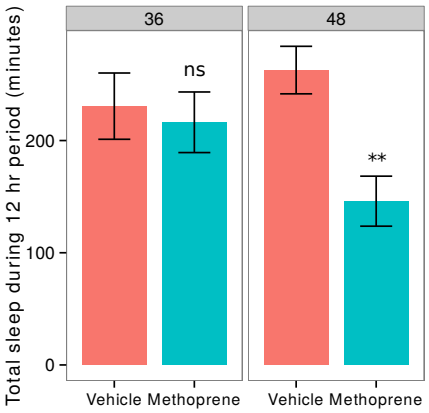


Figure 3.13: Methoprene increases starvation-induced sleep suppression. **(A)** A measurement of *Drosophila* sleep when fed methoprene, in percent of time asleep per hour. Light or dark regions represent whether experimental lighting was on or off. Flies were deprived of food between hours 24 and 48. **(B)** Total sleep for each genotype for the time period between either 24 and 36 hours **(36)**, or 36 and 48 hours **(48)** from the plot in **A**. Error bars in **B** and colored regions in **A** represent the standard error of the mean for each data point. n = 32 flies per condition. ** indicates significance versus controls with $p < 0.01$ by One-Way ANOVA followed by post-hoc Tukey HSD test, ns = not significant.

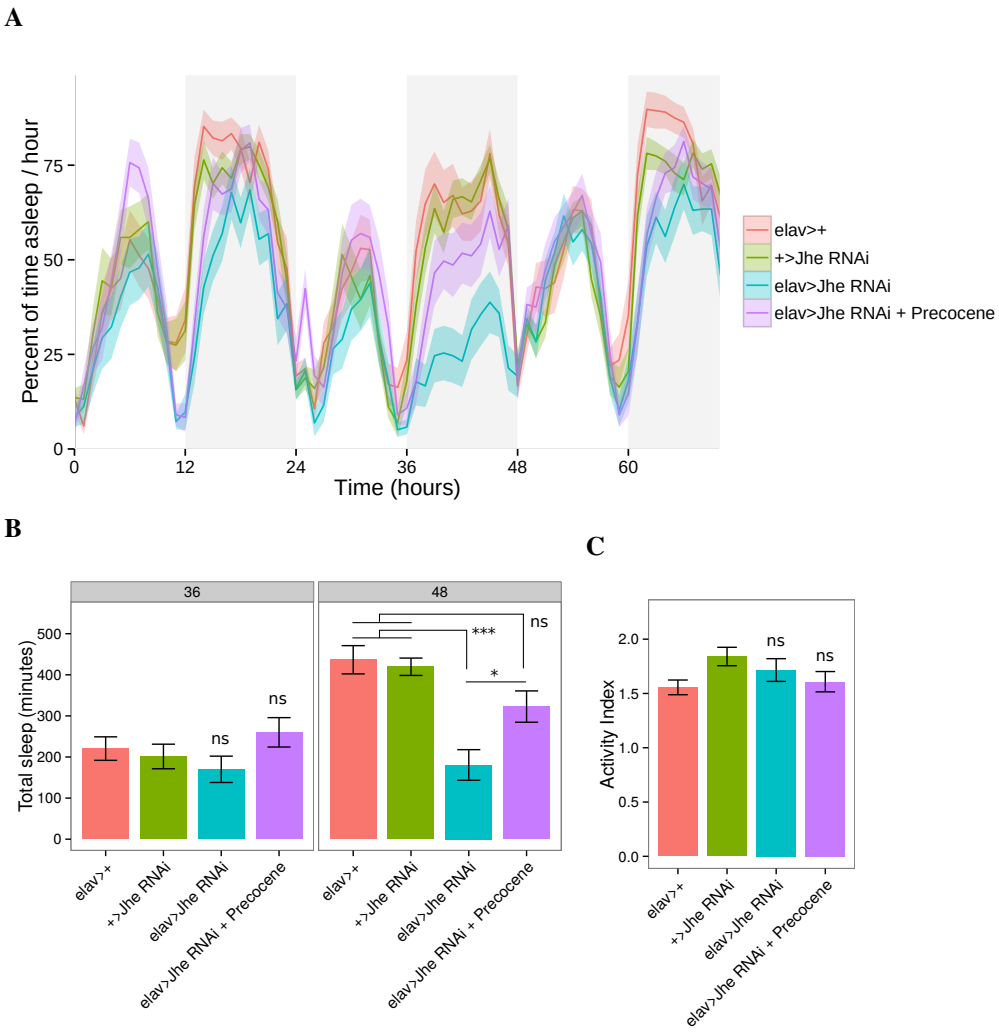


Figure 3.14: Precocene I rescues *Jhe* knockdown.
(A) A measurement of *Drosophila* sleep when fed precocene I, in percent of time asleep per hour. Light or dark regions represent whether experimental lighting was on or off. Flies were deprived of food between hours 24 and 48. **(B)** Total sleep for each genotype for the time period between either 24 and 36 hours **(36)**, or 36 and 48 hours **(48)** from the plot in **A**. **(C)** Average activity index (total activity normalized to time awake) for each condition for the time period between 36 and 48 hours from the plot in **A**. Error bars in **B/C** and colored regions in **A** represent the standard error of the mean for each data point. n = 24 flies per condition. * indicates significance versus controls with $p < 0.05$ by One-Way ANOVA followed by post-hoc Tukey HSD test, *** = $p < 0.001$, ns = not significant.

Chapter 4

Discussion

The original goal of this study was to identify a gene capable of controlling the behaviors associated with starvation. *Jhe* is a relatively unstudied gene not previously known to control behavior. What's more, *Jhe's* involvement in Juvenile Hormone metabolism suggests that these hormones play an important and novel role in the adult lifestage. Although this hormone is known to be present in the adult, no role for the hormone has been suggested aside from supporting oogenesis in females (Wilson et al., 1983; Shiao et al., 2008; Parthasarathy and Palli, 2011). I propose that *Jhe* regulation during starvation acts to coordinate changes in food intake and sleep. This occurs through its effects on Juvenile Hormone. The evidence for these assertions will be discussed here.

4.1 Regulation of *Jhe* is evolutionarily conserved

I originally identified *Jhe* as a conserved regulatory change using RNA sequencing in two separate, evolutionarily divergent insects. As discussed in Section 2.6, we used the definition of orthology established by Waterhouse et al. to match the genomes of *D. melanogaster* and *A. aegypti* at the gene-to-gene level. This approach was validated via statistical simulation- I obtained far more significantly regulated orthologous gene-pairs than would be expected if no conservation of regulatory changes had occurred. The correlation in gene expression between orthologs in each species was greater than zero (see Section 3.2.2). However, there

are two caveats with the form of statistical analysis used here. The statistical model purely predicts the total number of conserved changes expected if gene regulation was absolutely random in each species. As a result, the probability of each k value indicates the probability of obtaining a k value as extreme or more extreme under the model and cannot be used to validate the significance of any given orthologous pair (Jewell, 2014). Furthermore, calculating the given probability for individual orthologous pairs was deemed impractical, as calculating a specific p-value for every pair would require the calculation of probabilities for every possible model of conserved changes. No methodology exists with which to make these calculations (Jewell, 2014). With these caveats in mind, I am confident in the conclusion that gene regulatory changes are conserved during starvation between *D. melanogaster* and *A. aegypti*. Although we cannot measure the statistical significance of any individual orthogous conserved pair, *Jhe* is an excellent candidate for being a hunger-regulated gene in each species, based on the observed phenotypes in each organism as well as data from the literature.

Although both *Jhe* and its ortholog *AAEL005178* are significantly regulated by starvation, they are regulated in opposite directions. Whereas *Jhe* expression decreases (with a $\log_2$ fold-change of -0.84), the expression of its ortholog *AAEL005178*, actually increases, with a $\log_2$ fold-change of 1.01. Although initially perplexing, this difference in expression makes more sense with additional evidence. Both genes are predicted to function Juvenile Hormone esterases. JHE has been demonstrated to rapidly degrade all forms of JH, including JH III, JHB3, and MF (Campbell et al., 1998). If the expression of *Jhe* increases, JH titers are expected to decrease. If, on the other hand, *Jhe* expression decreases, JH titers will increase. Previous evidence indicates that the JH titers decrease in starved *Aedes aegypit* (Shiao et al., 2008; Hernandez-Martinez et al., 2015). This is consistent with an increase in expression of Juvenile Hormone esterase-type genes. Although we still lack evidence for an increase in JH titers upon starvation in *Drosophila*, the expression of *takeout* is consistent with an increase in Juvenile Hormone levels- it is downregulated when JH levels are high (Goodman and Cusson, 2012). Additionally, we found that exogenous application of methoprene phenocopied *Jhe* downregulation, demonstrating that an increase in JH signaling was consistent with our model of *Jhe* action (see Section 3.6). As mentioned in Section 4.4, I am currently working

on developing a method of definitively quantifying Juvenile Hormone titers during starvation in *Drosophila*.

4.2 *Jhe* affects sleep and feeding in adult *Drosophila*

Jhe is able to control behavior in adult *Drosophila*. Knockdown of *Jhe* resulted in increased feeding, and this was not due to a developmental defect (Figures 3.6 and 3.7). Interestingly, we found that this phenotype was not related to appetitive taste or caloric sensing of food, as consumption increased regardless of the food's palatability or nutritional value (Figure 3.6). At the same time, *Jhe* knockdown also caused an increase in starvation induced sleep suppression without affecting waking levels of activity (Figures 3.10 and 3.9B). These data are consistent with the behavioral and transcriptional changes that occur during starvation, as knockdown of *Jhe* reduces expression of the gene in a similar manner observed during hunger. Both sets of experiments relied upon neuronal knockdown of *Jhe*. Two different neuronal GAL4 drivers were used, indicating both that *Jhe* is expressed in neurons, as well as the conclusion that reducing the amount of *Jhe* expression in neurons is sufficient to elicit a behavioral change. *Jhe* has not been previously shown to act in this tissue.

4.3 *Jhe* exerts its effects through Juvenile Hormone metabolism

JHE is known to degrade all forms of JH in *Drosophila* as well as other organisms (Campbell et al., 1998; Goodman and Cusson, 2012). Additionally, JHE plays a greater role in JH degradation in adult *Drosophila* than the JHEH family of genes (as stated in Section 1.5.2.3, JHEHs are the other major pathway responsible for JH degradation) (Rauschenbach et al., 1995). Although JHEH's are more highly expressed, they are unable to degrade JHB3 with the same efficiency as JHE and cannot degrade MF (Casas et al., 1991). JHB3 and MF are the two most abundant JHs in *Drosophila* (Wen et al., 2015). All three *Drosophila* JHs are biologically active, and can bind and activate the known JH receptors (Shemshedini et al., 1990; Jones and Sharp, 1997; Godlewski et al., 2006; Wen et al., 2015). As mentioned before in Section 4.1, the most likely explanation for *Jhe's* behavioral phenotypes

is through its role in JH catabolism.

In Section 3.6, we demonstrate that methoprene is able to phenocopy the effects of *Jhe* knockdown. As mentioned before, methoprene is a highly specific and effective JH agonist able to bind and activate both the USP and MET-GCE receptors (Wilson and Fabian, 1986; Jones and Sharp, 1997). This means that methoprene is an effective method of simulating an increase in JH titers. Methoprene application has a number of advantages over other methods of JH application. Unlike JH III or other biological JH derivatives, methoprene is extremely stable and resistant to degradation, as it lacks the functional groups that JHE and JHEH act upon. This is especially important in light of the fact that addition of JH III has been shown to increase levels of proteins able to catabolize it (including JHE), possibly blunting any effect JH application might have (Kethidi et al., 2005). Nevertheless, when methoprene was added to wild-type flies, it resulted in an identical sleep phenotype observed during *Jhe* knockdown (Figure 3.13). This indicates that the result of neuronal *Jhe* knockdown is consistent with the predicted increase in JH titers this would generate. Even more convincingly, addition of precocene I, an anti-juvenoid agent, rescued the the phenotype caused by *Jhe* knockdown. Taken together, these data indicate that sleep suppression is controlled by JH levels in adult *Drosophila*. Although it is possible that the effects of *Jhe* and JH may be transduced through two parallel pathways that have the same phenotype, this is extremely unlikely. It has been demonstrated repeatedly that JHE makes a major contribution to the control of JH titers in *Drosophila* adults (Campbell et al., 1992; Rauschenbach et al., 1995).

4.4 Future work and directions

There are two key experiments that can be used to further reinforce the findings of this study. The first is a measurement of Juvenile Hormone titers in *Drosophila*. Although all evidence so far indicates that neuronal *Jhe* is controlling behavior by manipulating JH titers, we have so far not been able to show this directly. If we are able to demonstrate that hemolymph JH titers increase in starved versus fed *Drosophila* as well as show the same effect in fed neuronal *Jhe* knockdown flies versus controls, it will demonstrate that *Jhe* is manipulating JH levels. The alterna-

tive result (no change in hemolymph JH levels upon *Jhe* knockdown or starvation) is equally interesting, as it would imply that *Jhe* may be regulating JH activity on the local scale, either in or around JH target cells (rather than on a global scale, by changing hemolymph JH levels). Either way, this experiment would prove informative. I have made a large amount of progress towards this goal, and have prepared hemolymph extracts from all genotypes/conditions involved in the experiment. This extraction has also been confirmed to be effective, and these preparations contain detectable levels of JHs. Currently the only factor preventing JH quantification is the availability of an analytical standard for JHB3. After opting to quantify JH through multiple reaction monitoring on an LC-MS mass spectrometer, it proved impossible to develop a quantitative assay for JHB3- we have been unable to predict JHB3's breakdown products in the absence of a standard. Unfortunately, there seems to be no means of acquiring JHB3 either commercially or otherwise, so this experiment is stalled indefinitely until I am able to synthesize the compound.

The other experimental goal deals with identification of the JH targets able to effect changes in behavior. Although we have shown that *Jhe* is expressed in neurons, this gene's protein product is known to be secreted into the hemolymph. This means that JHE may act locally (through intracellular JH degradation), globally (by manipulation of hemolymph JH titers), or a combination of both. Despite this ambiguity, there are two excellent candidates for JH's behavioral targets.

The most obvious potential target of JH action is neurons. As discussed in Section 1.5.4, JH has previously been shown to act directly on neurons. This effect is rapid, inducing short-term neuronal depression in as little as 2 minutes (Richter and Gronert, 1999). Although this is an exciting possibility, the behavioral assays used in this study do not have the temporal resolution required to resolve effects on this timescale.

Another possible target of JH action may be the fat body. The fat body is roughly equivalent to mammalian adipose tissue, and has previously been shown to both act as a nutrient sensor and is capable of signalling to the brain to effect behavior (Rajan and Perrimon, 2012). In times of starvation, the fat body induces insulin-like peptide release by signalling to the brain through the *Drosophila* leptin, *upd2* (Rajan and Perrimon, 2012). Insulin signalling has previously been shown

to affect feeding behavior, and this action occurs on a similar timescale to that observed in this study (Stafford et al., 2012). The fat body has also previously been shown to respond to JH levels, making this tissue a particularly attractive possible target of JH action (Shiao et al., 2008; Parthasarathy and Palli, 2011).

4.5 Final remarks

As stated previously, the goal of this study was to identify and characterize a novel regulator of hunger-induced behavior. I believe that this goal has been accomplished. *Jhe* is able to control feeding and sleep suppression through its effects on Juvenile Hormone titers. In addition, this study demonstrated that Juvenile Hormone levels are able to control behavior in the adult, and this occurs in a physiologically-relevant manner. JHs were not previously known to act in this manner. *Jhe* downregulation in starved *Drosophila* likely increases JH levels, inducing behaviors that should help hungry flies survive like increased food consumption. Juvenile Hormone esterase regulation also occurs in starved *Aedes aegypti*, raising the possibility that this is an important regulatory change that occurs in a large number of insect species upon starvation.

These conclusions have a number of economic implications, with immediate application to modern pest control. Methoprene is widely used to control mosquito populations around the world due to its high specificity and low toxicity. Given our finding that methoprene (and JHs in general) can control behavior, it is worth examining the effects of JH feeding on economically and environmentally critical species like honeybees. If methoprene deployment proves disruptive to these species, it may be worth reconsidering the use of Juvenile Hormone-based insecticides in regions where protection of these species is paramount.

Bibliography

Anders, S., Pyl, P. T., and Huber, W. (2014). HTSeq - A Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)*, 31(2):166–169. → pages 25

Andrews, P., Bokil, H., Kaur, S., Loader, C., Maniar, H., Mehta, S., Mitra, P. P., Nalatore, H., Yadav, R., and Shukla, R. (2015). Chronux. → pages 72

Arrese, E. and Soulages, J. (2010). Insect Fat Body: Energy, Metabolism, and Regulation. *Annual review of entomology*, 55:207–225. → pages 20, 21

Baumann, A., Barry, J., Wang, S., Fujiwara, Y., and Wilson, T. G. (2010). Paralogous genes involved in juvenile hormone action in Drosophila melanogaster. *Genetics*, 185(4):1327–36. → pages 19

Bernardo, T. J. and Dubrovsky, E. B. (2012). Molecular Mechanisms of Transcription Activation by Juvenile Hormone: A Critical Role for bHLH-PAS and Nuclear Receptor Proteins. *Insects*, 3(4):324–338. → pages 11

Bonning, B. C., Booth, T. F., and Hammock, B. D. (1997). Mechanistic Studies of the Degradation of Juvenile Hormone Esterase in Manduca sexta. *Archives of Insect Biochemistry and Physiology*, 286(91):275–286. → pages 17

Bowers, W. S., Ohta, T., Cleere, J. S., and Marsella, P. A. (1976). Discovery of Anti-Juvenile Hormones in Plants. *Science*, 193(4253):542–547. → pages 18

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, 415:401–415. → pages 6, 7

Campbell, P. M., Healy, M. J., and Oakeshott, J. G. (1992). Characterization of Juvenile Hormone Esterase in Drosophila melanogaster. *Insect Biochemistry and Molecular Biology*, 22(7):665–677. → pages 17, 57

Campbell, P. M., Oakeshott, J. G., and Healy, M. J. (1998). Purification and kinetic characterisation of juvenile hormone esterase from Drosophila melanogaster. *Insect Biochemistry and Molecular Biology*, 28(7):501–515. → pages 16, 17, 48, 55, 56

Casas, J., Harshman, L. G., Messeguer, A., Kuwano, E., and Hammock, B. D. (1991). In Vitro Metabolism of Juvenile Hormone Ill and Juvenile Hormone Ill Bisepoxide by Drosophila melanogaster and Mammalian Cytosolic Epoxide Hydrolase. *Archives of Biochemistry and Biophysics*, 286(1):153–158. → pages 17, 56

Celniker, S. E., Dillon, L. A. L., Gerstein, M. B., Gunsalus, K. C., Henikoff, S., Karpen, G. H., Kellis, M., Lai, E. C., Lieb, J. D., Macalpine, D. M., Micklem, G., Piano, F., Snyder, M., Stein, L., White, K. P., and Waterston, R. H. (2009). Unlocking the secrets of the genome. *Nature*, 459(June):927–930. → pages 16, 17

Chamseddin, K. H., Khan, S. Q., Nguyen, M. L. H., Antosh, M., Morris, S. N. S., Kolli, S., Neretti, N., Helfand, S. L., and Bauer, J. H. (2012). takeout-dependent longevity is associated with altered Juvenile Hormone signaling. *Mechanisms of ageing and development*, 133(11-12):637–46. → pages 15

Chu, B., Chui, V., Mann, K., and Gordon, M. D. (2014). Presynaptic Gain Control Drives Sweet and Bitter Taste Integration in Drosophila. *Current Biology*, 24(17):1978–1984. → pages 2

Crone, E. J., Sutherland, T. D., Campbell, P. M., Coppin, C. W., Russell, R. J., and Oakeshott, J. G. (2007). Only one esterase of Drosophila melanogaster is likely to degrade juvenile hormone in vivo. *Insect biochemistry and molecular biology*, 37(6):540–9. → pages 16, 39, 48

dos Santos, G., Schroeder, A. J., Goodman, J. L., Strelets, V. B., Crosby, M. A., Thurmond, J., Emmert, D. B., and Gelbart, W. M. (2015). FlyBase: introduction of the Drosophila melanogaster Release 6 reference genome assembly and large-scale migration of genome annotations. *Nucleic Acids Research*, 43(D1):D690–D697. → pages 6

Dubrovsky, E. B., Dubrovskaya, V. A., Bernardo, T., Otte, V., Difilippo, R., and Bryan, H. (2011). The Drosophila FTZ-F1 Nuclear Receptor Mediates Juvenile Hormone Activation of E75A Gene Expression through an Intracellular Pathway. *Journal of Biological Chemistry*, 286(38):33689–33700. → pages 19

Duffy, J. B. (2002). GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis (New York, N.Y. : 2000)*, 34(1-2):1–15. → pages 7

Durinck, S., Moreau, Y., Kasprzyk, A., Davis, S., De Moor, B., Brazma, A., and Huber, W. (2005). BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics (Oxford, England)*, 21(16):3439–40. → pages 6, 25

Dus, M., Ai, M., and Suh, G. S. B. (2013). Taste-independent nutrient selection is mediated by a brain-specific Na(+)/solute co-transporter in Drosophila. *Nature neuroscience*, 16(5):526–8. → pages 2

Farhadian, S. F., Suárez-Fariñas, M., Cho, C. E., Pellegrino, M., and Vosshall, L. B. (2012). Post-fasting olfactory, transcriptional, and feeding responses in Drosophila. *Physiology & behavior*, 105(2):544–53. → pages 2, 3, 31, 33

Flicek, P., Amode, M. R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S., Gil, L., Giron, C. G., Gordon, L., Hourlier, T., Hunt, S., Johnson, N., Juettemann, T., Kahari, A. K., Keenan, S., Kulesha, E., Martin, F. J., Maurel, T., McLaren, W. M., Murphy, D. N., Nag, R., Overduin, B., Pignatelli, M., Pritchard, B., Pritchard, E., Riat, H. S., Ruffier, M., Sheppard, D., Taylor, K., Thormann, A., Trevanion, S. J., Vullo, A., Wilder, S. P., Wilson, M., Zadissa, A., Aken, B. L., Birney, E., Cunningham, F., Harrow, J., Herrero, J., Hubbard, T. J. P., Kinsella, R., Muffato, M., Parker, A., Spudich, G., Yates, A., Zerbino, D. R., and Searle, S. M. J. (2014). Ensembl 2014. *Nucleic Acids Research*, 42(D1):D749–D755. → pages 6

Flood, T. F., Iguchi, S., Gorczyca, M., White, B., Ito, K., and Yoshihara, M. (2013). A single pair of interneurons commands the Drosophila feeding motor program. *Nature*, 499(7456):83–7. → pages 2

Fujikawa, K., Takahashi, A., Nishimura, A., Itoh, M., Takano-Shimizu, T., and Ozaki, M. (2009). Characteristics of genes up-regulated and down-regulated after 24 h starvation in the head of Drosophila. *Gene*, 446(1):11–7. → pages 2, 3, 31, 33

Gilestro, G. F. and Cirelli, C. (2009). pySolo: a complete suite for sleep analysis in Drosophila. *Bioinformatics (Oxford, England)*, 25(11):1466–7. → pages 30

Giraldo-Calderon, G. I., Emrich, S. J., MacCallum, R. M., Maslen, G., Dialynas, E., Topalis, P., Ho, N., Gesing, S., Madey, G., Collins, F. H., and Lawson, D. (2015). VectorBase: an updated bioinformatics resource for invertebrate

vectors and other organisms related with human diseases. *Nucleic Acids Research*, 43(D1):D707–D713. → pages 8

Godlewski, J., Wang, S., and Wilson, T. G. (2006). Interaction of bHLH-PAS proteins involved in juvenile hormone reception in Drosophila. *Biochemical and biophysical research communications*, 342(4):1305–11. → pages 19, 56

Goodman, W. G. and Cusson, M. (2012). The Juvenile Hormones. In Gilbert, L., editor, *Insect Endocrinology*, pages 311–365. Elsevier B.V. → pages 14, 15, 18, 19, 20, 21, 55, 56

Gordon, M. D. and Scott, K. (2009). Motor control in a Drosophila taste circuit. *Neuron*, 61(3):373–84. → pages 2

Harmon, M. A., Boehmt, M. F., Heymant, R. A., and Mangelsdorf, D. J. (1995). Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. *Proceedings of the National Academy of Sciences*, 92(June):6157–6160. → pages 19

Hendricks, J. C., Finn, S. M., Panckeri, K., Chavkin, J., Williams, J., Sehgal, A., and Pack, A. (2000). Rest in Drosophila Is a Sleep-like State. *Neuron*, 25(1):129–138. → pages 30

Hernandez-Martinez, S., Rivera-Perez, C., Nouzova, M., and Noriega, F. G. (2015). Coordinated changes in JH biosynthesis and JH hemolymph titers in Aedes aegypti mosquitoes. *Journal of insect physiology*, 72:22–7. → pages 55

Hong, S.-H., Lee, K.-S., Kwak, S.-J., Kim, A.-K., Bai, H., Jung, M.-S., Kwon, O.-Y., Song, W.-J., Tatar, M., and Yu, K. (2012). Minibrain/Dyrk1a regulates food intake through the Sir2-FOXO-sNPF/NPY pathway in Drosophila and mammals. *PLoS genetics*, 8(8):e1002857. → pages 14

Ichinose, R., Nakamura, A., Yamoto, T., Booth, T. I. M. F., Maeda, S., Hammocki, B. D., Road, M., Ox, O., and Toxicology, E. (1992). Uptake of Juvenile Hormone Esterase by Pericardial Cells of Manduca sexta. *Insect biochemistry and molecular biology*, 22(8):893–904. → pages 16, 17

Illumina (2011). RNA-Seq Data Comparison with Gene Expression Microarrays. *White Paper*. → pages 3

Ishimoto, H. and Kitamoto, T. (2010). The steroid molting hormone Ecdysone regulates sleep in adult Drosophila melanogaster. *Genetics*, 185(1):269–81. → pages 19

Iwema, T., Chaumot, A., Studer, R. a., Robinson-Rechavi, M., Billas, I. M. L., Moras, D., Laudet, V., and Bonneton, F. (2009). Structural and evolutionary innovation of the heterodimerization interface between USP and the ecdysone receptor ECR in insects. *Molecular biology and evolution*, 26(4):753–68. → pages 19

Ja, W. W., Carvalho, G. B., Mak, E. M., de la Rosa, N. N., Fang, A. Y., Liong, J. C., Brummel, T., and Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. *Proceedings of the National Academy of Sciences of the United States of America*, 104(20):8253–6. → pages 10, 28

Jewell, S. (2014). Measuring Conserved Changes in Gene Regulation. Technical report. → pages 55

Jones, G., Jones, D., Li, X., Tang, L., Ye, L., Teal, P., Riddiford, L., Sandifer, C., Borovsky, D., and Martin, J.-R. (2010). Activities of natural methyl farnesoids on pupariation and metamorphosis of Drosophila melanogaster. *Journal of Insect Physiology*, 56(10):1456–1464. → pages 19

Jones, G. and Sharp, P. (1997). Ultraspiracle : An invertebrate nuclear receptor for juvenile hormones. *Proceedings of the National Academy of Sciences*, 94(December):13499–13503. → pages 19, 56, 57

Kain, P. and Dahanukar, A. (2015). Secondary Taste Neurons that Convey Sweet Taste and Starvation in the Drosophila Brain. *Neuron*, 85(4):819–832. → pages 2

Keene, A. C., Duboué, E. R., McDonald, D. M., Dus, M., Suh, G. S. B., Waddell, S., and Blau, J. (2010). Clock and cycle limit starvation-induced sleep loss in Drosophila. *Current biology : CB*, 20(13):1209–15. → pages 2, 10, 44, 45, 51

Kethidi, D. R., Xi, Z., and Palli, S. R. (2005). Developmental and hormonal regulation of juvenile hormone esterase gene in Drosophila melanogaster. *Journal of insect physiology*, 51(4):393–400. → pages 16, 57

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology*, 14(4):R36. → pages 25

Kume, K., Kume, S., Park, S. K., Hirsh, J., and Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(32):7377–84. → pages 30

Langmead, B. and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods*, 9(4):357–9. → pages 25

Li, W.-c. and Riddiford, L. M. (1996). Differential Expression of the Two Duplicated Insecticyanin Genes , ins-a and ins-b , in the Black Mutant of Manduca sexta. *Archives of Biochemistry and Biophysics*, 330(1):65–70. → pages 20

Linkert, M., Rueden, C. T., Allan, C., Burel, J.-M., Moore, W., Patterson, A., Loranger, B., Moore, J., Neves, C., Macdonald, D., Tarkowska, A., Sticco, C., Hill, E., Rossner, M., Eliceiri, K. W., and Swedlow, J. R. (2010). Metadata matters: access to image data in the real world. *The Journal of cell biology*, 189(5):777–82. → pages 69

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12):550. → pages 25

Mathworks (2015). Video Stabilization Using Point Feature Matching. → pages 71

McGuire, S. E., Mao, Z., and Davis, R. L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. *Science's STKE : signal transduction knowledge environment*, 2004(220):pl6. → pages 7

Meunier, N., Belgacem, Y. H., and Martin, J.-R. (2007). Regulation of feeding behaviour and locomotor activity by takeout in Drosophila. *The Journal of experimental biology*, 210(Pt 8):1424–34. → pages 15, 40

Miyamoto, T., Slone, J., Song, X., and Amrein, H. (2012). A fructose receptor functions as a nutrient sensor in the Drosophila brain. *Cell*, 151(5):1113–25. → pages 2, 22

Noriega, F. G. (2014). Juvenile Hormone Biosynthesis in Insects: What Is New, What Do We Know, and What Questions Remain? *International Scholarly Research Notices*, 2014:1–16. → pages 12, 13

Organization, W. H. (2008). Guidelines for Drinking-Water Quality, 3rd edition including 1st and 2nd addenda. Technical report. → pages 20, 49

Parthasarathy, R. and Palli, S. R. (2011). Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, Tribolium castaneum. *Insect biochemistry and molecular biology*, 41(5):294–305. → pages 21, 54, 59

Rajan, A. and Perrimon, N. (2012). Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. *Cell*, 151(1):123–37. → pages 21, 58

Rauschenbach, I., Khlebodarova, T., Chentsova, N., Gruntenko, N., Grenback, L., Yantsen, E., and Filipenko, M. (1995). Metabolism of the juvenile hormone in Drosophila adults under normal conditions and heat stress: Genetical and biochemical aspects. *Journal of Insect Physiology*, 41(2):179–189. → pages 17, 56, 57

Restifo, L. L. and Wilson, T. G. (1998). A Juvenile Hormone Agonist Reveals Distinct Developmental Pathways Mediated by Ecdysone-Inducible Broad Complex Transcription. *Developmental Genetics*, 159(January):141–159. → pages 27

Richter, K. and Gronert, M. (1999). Neurotropic effect of juvenile hormone III in larvae of the cockroach, Periplaneta americana. *Journal of Insect Physiology*, 45(12):1065–1071. → pages 21, 58

Robertson, G., Schein, J., Chiu, R., Corbett, R., Field, M., Jackman, S. D., Mungall, K., Lee, S., Okada, H. M., Qian, J. Q., Griffith, M., Raymond, A., Thiessen, N., Cezard, T., Butterfield, Y. S., Newsome, R., Chan, S. K., She, R., Varhol, R., Kamoh, B., Prabhu, A.-L., Tam, A., Zhao, Y., Moore, R. A., Hirst, M., Marra, M. A., Jones, S. J. M., Hoodless, P. A., and Birol, I. (2010). De novo assembly and analysis of RNA-seq data. *Nat Meth*, 7(11):909–912. → pages 4

Root, C. M., Ko, K. I., Jafari, A., and Wang, J. W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. *Cell*, 145(1):133–44. → pages 14

Sarov-blat, L., So, W. V., Liu, L., Rosbash, M., and Hughes, H. (2000). The Drosophila takeout Gene Is a Novel Molecular Link between Circadian Rhythms and Feeding Behavior. *Cell*, 101:647–656. → pages 15, 40

Schmid, B., Helfrich-Forster, C., and Yoshii, T. (2011). A new ImageJ plug-in "ActogramJ" for chronobiological analyses. *Journal of biological rhythms*, 26(5):464–7. → pages 30

Semmelhack, J. L. and Wang, J. W. (2009). Select Drosophila glomeruli mediate innate olfactory attraction and aversion. *Nature*, 459(7244):218–23. → pages 1

Shemshedini, L., Lanoue, M., and Wilson, T. G. (1990). Evidence for a Juvenile Hormone Receptor Involved in Protein Synthesis in Drosophila melanogaster. *The Journal of Biological Chemistry*, 265(4):1913–1918. → pages 18, 56

Shiao, S.-H., Hansen, I. a., Zhu, J., Sieglaff, D. H., and Raikhel, A. S. (2008). Juvenile hormone connects larval nutrition with target of rapamycin signaling in the mosquito Aedes aegypti. *Journal of insect physiology*, 54(1):231–9. → pages 21, 54, 55, 59

Stafford, J. W., Lynd, K. M., Jung, A. Y., and Gordon, M. D. (2012). Integration of Taste and Calorie Sensing in Drosophila. *The Journal of Neuroscience*, 32(42):14767–14774. → pages 2, 10, 28, 59

Stout, J., Atkins, G., and Zacharias, D. (1991). Regulation of cricket phonotaxis through hormonal control of the threshold of an identified auditory neuron. *Journal of comparative physiology.*, 169:765–772. → pages 21

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotech*, 28(5):511–515. → pages 4

Wang, X.-h., Aliyari, R., Li, W.-x., Li, H.-w., Kim, K., Atkinson, P., and Ding, S.-w. (2006). RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. *Science*, 312(5772):452–454. → pages 7

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, 10(1):57–63. → pages 3

Waterhouse, R. M., Tegenfeldt, F., Li, J., Zdobnov, E. M., and Kriventseva, E. V. (2013). OrthoDB: a hierarchical catalog of animal, fungal and bacterial orthologs. *Nucleic acids research*, 41(Database issue):D358–65. → pages 9, 25, 36, 39, 54

Wen, D., Rivera-Perez, C., Abdou, M., Jia, Q., He, Q., Liu, X., Zyaan, O., Xu, J., Bendena, W. G., Tobe, S. S., Noriega, F. G., Palli, S. R., Wang, J., and Li, S. (2015). Methyl farnesoate plays a dual role in regulating Drosophila metamorphosis. *PLoS genetics*, 11(3):e1005038. → pages 13, 14, 17, 56

Wheeler, D. E. and Nijhout, H. F. (1981). Soldier determination in ants: new role for juvenile hormone. *Science*, 213:361–363. → pages 21

Williams, D. W. and Truman, J. W. (2005). Remodeling dendrites during insect metamorphosis. *Journal of neurobiology*, 64(1):24–33. → pages 21

Wilson, T. G. and Ashok, M. (1998). Insecticide resistance resulting from an absence of target-site gene product. *Proceedings of the National Academy of Sciences*, 95(November):14040–14044. → pages 18

Wilson, T. G. and Fabian, J. (1986). A Drosophila melanogaster Mutant Resistant to a Chemical Analog of Juvenile Hormone. *Developmental Biology*, 201:190–201. → pages 18, 57

Wilson, T. G., Landers, M. H., and Happ, G. M. (1983). Precocene I and II inhibition of vitellogenic oocyte development in Drosophila melanogaster. *Journal of insect physiology*, 29(3):249 – 254. → pages 51, 54

Wyatt, G. (1961). The Biochemistry of Insect Hemolymph. *Annual Review of Entomology*, 6:75–102. → pages 31

Yarmolinsky, D. a., Zuker, C. S., and Ryba, N. J. P. (2009). Common sense about taste: from mammals to insects. *Cell*, 139(2):234–44. → pages 1

Yin, C.-M., Zou, B.-X., Jiang, M., Li, M.-f., Qin, W., Potter, T., and Stoffolano, J. (1995). Identification of Juvenile Hormone III Bisepoxide (JHB3) , Juvenile Hormone III and Methyl Farnesoate Secreted by the Corpus Allatum of Phormia regina (Meigen), In Vitro and Function of JHB3 Either Applied Alone or as a Part of a Juvenoid Blend. *Journal of insect physiology*, 41(6):473–439. → pages 17

Appendix A

Supporting Materials

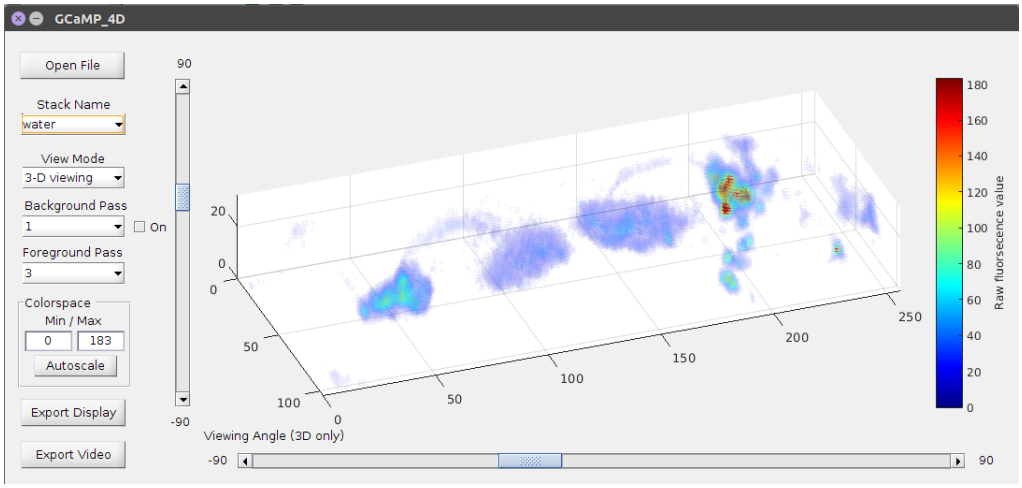
Although not immediately relevant to the subject of this thesis, I developed two pieces of software in addition to the `actmon` R package that have seen relatively heavy use by both my and other labs. These are covered briefly here.

A.1 `GCaMP_4D`

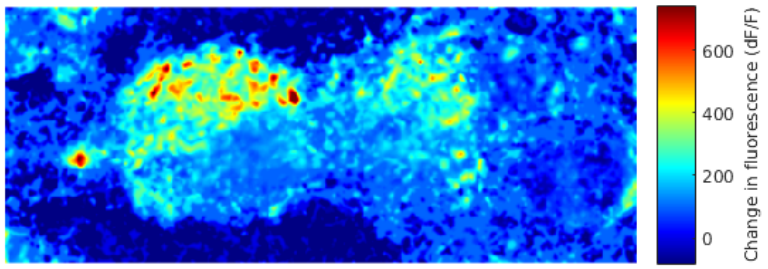
`GCaMP_4D` allows researchers to view and analyze microscopy data in a truly 3D manner, allowing you to use older equipment like confocal microscopes in a manner that has previously only been possible with newer equipment (like light-sheet microscopes). As an example, the activity of a 3D field of neurons (expressing a calcium sensor) can be imaged in real time by passing a microscopy plane repeatedly through the same 3D zone. Whereas traditional data analysis (or the human eye) might be unable to find changes in fluorescence between two timepoints, `GCaMP_4D` is much more sensitive, and considers the entire 3D field, meaning that one feature cannot "obscure" another. Researchers can even make videos of the entire 3D space over time or choose to focus on a single slice of the composite field through the integrated user interface.

The expected input is any microscopy file that can be opened using Bio-Formats (Linkert et al., 2010). Using metadata included in the file, the stack is formatted into a series of 3D images, each representing one "pass" through the specimen. Passes can be viewed in either 2D or 3D. When viewing in 2D, each pass is "flat-

A



B



C

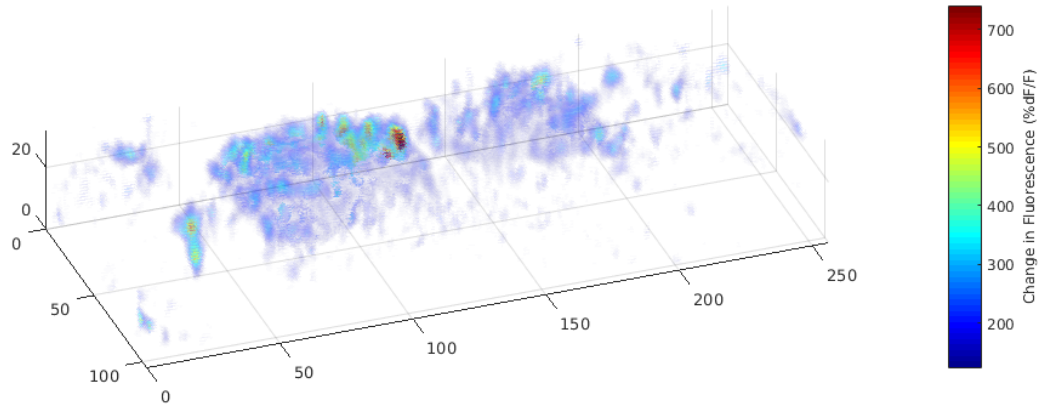


Figure A.1: Sample `GCaMP_4D` output.
A scaled down image of `GCaMP_4D`'s user interface is displayed in **A**. **B** and **C** show the change in fluorescence of the sample from **A** in 2D (**B**) or 3D (**C**) respectively. The sample being viewed is a pair of *Drosophila* gustatory neurons responding to stimuli.

tened" to a single maximum projection (each pixel is the vertical maximum of all the pixels at that position in the stack) or simply viewed as an individual microscope image at a specified depth.

Importantly, this algorithm is able to perform 3D field subtraction. An entire 3D foreground pass can be compared to a 3D background pass. To make this possible, every image in each Z-stack/pass are first "stabilized" using the SURF/MSAC algorithms in a similar methodology to that demonstrated by Mathworks (2015). This ensures that the features in each pass actually line up, even though the specimen may have shaken or moved during imaging. Once stabilization is complete, the difference between the images is computed and displayed back to the user after gaussian denoising. This 3D field subtraction algorithm is applied regardless of whether or not a researcher is viewing a sample in 2D or 3D. The formula for image subtraction is as follows (occurs on a per-pixel basis where division by zero artifacts converted to zero):

$$\frac{foreground - background}{background} * 100 \tag{A.1}$$

This tool exports both still images and videos of the region being imaged for later use. It is intended as both a general 3D viewer and full analysis tool, and is best used for identifying changes in fluorescence in over the course of a single imaging session. Currently this tool has been used to help identify and characterize a set of second-order *Drosophila* gustatory neurons, although it also has other applications including simply viewing a confocal Z-stack in three dimensions. The MATLAB source code and standalone binary executables for Windows, OSX, and Linux are available at https://github.com/kazi11/GCaMP_4D.

A.2 `fly_tracker`

This is a collection of MATLAB algorithms designed to track and quantify the behavior of individual *Drosophila* adults and larvae. There are two separate algorithms for tracking larvae and adults. The larval tracking script is optimized for slowly-moving animals and tracks individual objects after background subtraction. The adult fly tracking script tracks adult flies by finding the darkest pixel after background division, and calculates the centroid (i.e. where is the middle

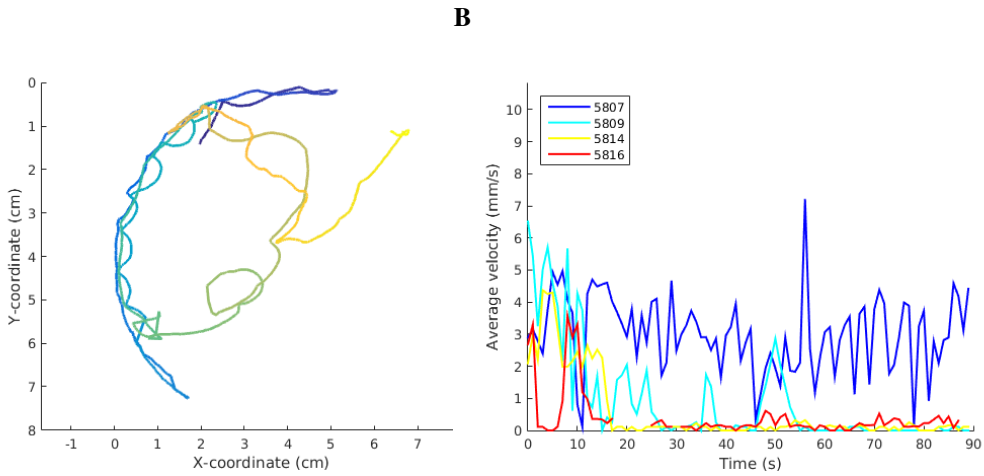


Figure A.2: Sample `fly_tracker` output.
(A) A sample position trace calculated from a cellphone video of an adult fly. Blue represents the fly's position at the beginning of the video and yellow represents the fly's final position. **(B)** Velocities calculated from several fly position traces.

of the darker pixels) of the surrounding region and is loosely based upon FTrack (Andrews et al., 2015). Both scripts are able to intelligently identify false tracks and interpolate missing data. The output of both scripts can be used for any of the downstream analysis tools and can generate robust tracks from even cellphone-quality video.

Downstream analysis is quite straightforward. The position traces created by the fly and larvae trackers can be used to calculate a number of different statistics, including velocity, distance traveled, and probability of an animal being in a given part of the experimental arena. Output from these scripts can then be plotted and statistics are automatically performed between different genotypes/experimental conditions.