Expression Analysis of Cytoskeletal and Ribosomal Genes during Adult Diapause in the Northern House Mosquito, *Culex pipiens*

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Mijung Kim, M.S.

Graduate Program in Entomology

The Ohio State University

2009

Dissertation Committee:

Professor David L. Denlinger, Advisor
Professor Susan W. Fisher
Professor Glen R. Needham
Professor Peter J. Reiser
ABSTRACT

The northern house mosquito *Culex pipiens* L., a vector of arthropod-borne viruses, is capable of entering an overwintering dormancy known as diapause. Adult diapause in *C. pipiens* is regulated at environmental, hormonal, and molecular levels. In this thesis, select gene groups including cytoskeletal genes and genes encoding ribosomal proteins (rps) were cloned and their expression patterns and function related to diapause were examined.

The gene in *C. pipiens* that encodes beta-tubulin, a major cytoskeletal gene, has high similarity with *beta-tubulins* from other insects. This gene was up-regulated in thoracic muscles (mostly flight muscles) of nondiapausing female mosquitoes but was down-regulated during diapause. Similarly, microtubule abundance was significantly lower in thoracic muscles of diapausing females than in thoracic muscles from nondiapausing females. Microtubule abundance also declined in midguts of nondiapausing females after low temperature exposure. However, in diapausing females, microtubule abundance was already low and remained at a low
level even after low temperature exposure. These results showing lower expression of *beta-tubulin* and microtubule abundance in the thoracic muscles suggest a link to the reduced flight activity during diapause and in response to low temperature.

Two ribosomal proteins located on a small ribosomal subunits, *rpS3a* and *rpS2*, are proposed to function in suppressing ovarian development during adult diapause in *C. pipiens* based on RNA interference (RNAi) results. *RpS3a* was continuously expressed in nondiapausing females but was less expressed for a brief period 7-10 days after adult eclosion in diapausing females. When RNAi was directed against *rpS3a* in nondiapausing females ovarian development was halted as it is in diapause. The effect persisted for approximately 10 days. Application of juvenile hormone III, an endocrine trigger known to terminate diapause, rescued the arrested ovarian development caused by dsrpS3a injection.

*RpS2* also has a potential role in regulating reproductive diapause in *C. pipiens*. *RpS2* is expressed strongly from 1 day to 1 month in nondiapausing females, whereas it is down-regulated on day 5-18 in diapausing females. RNAi directed against rpS2 suppressed the growth of follicles in nondiapausing female mosquitoes, and topical JHIII application again was able to rescue the arrested follicles. *RpL19*
was used as a control ribosomal protein, and the injected RNAi directed against
rpL19 did not affect the arrest of ovarian development. Thus, the results suggest that
rpS3a and rpS2 are ribosomal proteins that play a role in regulating the ovarian
development related to diapause in this species.

Another ribosomal protein, rpS6, was examined in relationship to heat shock
protein 90 (hsp90) in nondiapausing and diapausing female mosquitoes. Both rpS6
and hsp90 were continuously expressed in nondiapausing females, but they were
synchronously down-regulated around day 14 after adult eclosion in diapausing
females. RNAi directed against hsp90 was performed to evaluate a possible
correlation with rpS6. When RNAi was directed against hsp90 in nondiapausing
females, rpS6, as well as hsp90, was suppressed. These results suggest that the
down-regulation of hsp90 in diapausing mosquitoes contributes to the low
expression of rpS6 during diapause.

These experiments, thus, define several genes that appear to be critical for the
overwintering diapause in this important disease vector.
Dedicated to Jeongwoo
ACKNOWLEDGMENTS

I would like to express my gratitude to all those who gave me the possibility to complete these projects and my thesis.

I would like to thank my advisor and mentor, David L. Denlinger, Ph.D., for his endless support to pursue my projects and for his encouragement.

I am pleased to thank my committee members, Susan W. Fisher, Ph.D., Glen R. Needham, Ph.D., and Peter J. Reiser, Ph.D. for their interests in my work and for their valuable advices and comments.

I am grateful to previous and current members at Denlinger’s lab, especially Josh B. Benoit and Cheolho Sim.

Also, I would like to give special thanks to my husband Jeongwoo Han and my sister Eunha Kim.
VITA

May 10, 1979……………………..……..Born - Seoul, S.Korea

2003…………………………………..…Bachelor of Science, Genetic Engineering
   Sungkyunkwan University

2004 – 2006…………………………..…Graduate Research Associate
   The Ohio State University

2006………………………………..……Master of Science, Entomology
   The Ohio State University

2006 – present………………………….Graduate Teaching and Research Associate
   The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Entomology
TABLE OF CONTENTS

Abstract .................................................................................................................. ii
Dedication ............................................................................................................. v
Acknowledgments ................................................................................................ vi
Vita ......................................................................................................................... vii
List of Figures ........................................................................................................ x

Chapters:
1. Introduction ........................................................................................................... 1
   References ............................................................................................................. 8

2. Decrease in expression of beta-tubulin and microtubule abundance
   in flight muscles during diapause in adults of Culex pipiens ................................ 12
   Abstract ............................................................................................................. 12
   Introduction ....................................................................................................... 13
   Materials and Methods ....................................................................................... 15
   Results ................................................................................................................. 19
   Discussion .......................................................................................................... 23
   References ........................................................................................................... 27

3. RNA interference directed against ribosomal protein S3a suggests
   a link between this gene and arrested ovarian development during adult diapause
   in Culex pipiens .............................................................................................. 37
   Abstract ............................................................................................................. 37
   Introduction ....................................................................................................... 38
   Materials and Methods ....................................................................................... 40
   Results ................................................................................................................. 44
   Discussion .......................................................................................................... 47
   References ........................................................................................................... 51
4. A potential role for ribosomal protein S2 in the gene network regulating reproductive diapause in the mosquito *Culex pipiens*…………………………………….62
   Abstract………………………………………………………………62
   Introduction…………………………………………………………63
   Materials and Methods…………………………………………65
   Results………………………………………………………………70
   Discussion…………………………………………………………..72
   References………………………………………………………….76

5. Interaction between heat shock protein 90 and ribosomal protein S6 in the northern house mosquito, *Culex pipiens*……………………………………..88
   Abstract……………………………………………………………..88
   Introduction……………………………………………………….89
   Materials and Methods…………………………………………91
   Results………………………………………………………………94
   Discussion………………………………………………………….96
   References…………………………………………………………..98

6. Conclusions…………………………………………………………………….104

Appendix: Ribosomal protein S13 (rpS13) induced by low temperature and influenced during diapause in the northern house mosquito, *Culex pipiens*……109
   Introduction…………………………………………………………109
   Materials and Methods…………………………………………110
   Results and Discussion…………………………………………112
   References…………………………………………………………..115

Bibliography………………………………………………………………………119
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>32</td>
</tr>
<tr>
<td>2.2</td>
<td>33</td>
</tr>
<tr>
<td>2.3</td>
<td>34</td>
</tr>
<tr>
<td>2.4</td>
<td>35</td>
</tr>
<tr>
<td>2.5</td>
<td>36</td>
</tr>
<tr>
<td>3.1</td>
<td>56</td>
</tr>
<tr>
<td>3.2</td>
<td>57</td>
</tr>
<tr>
<td>3.3</td>
<td>58</td>
</tr>
<tr>
<td>3.4</td>
<td>60</td>
</tr>
<tr>
<td>3.5</td>
<td>61</td>
</tr>
<tr>
<td>4.1</td>
<td>80</td>
</tr>
<tr>
<td>4.2</td>
<td>82</td>
</tr>
<tr>
<td>4.3</td>
<td>83</td>
</tr>
</tbody>
</table>
4.4 Primary follicles from nondiapausing and diapausing females stained with neutral red……………………………………………………………………………….85

4.5 JHIII rescues the suppression of oogenesis caused by dsrpS2……………87

5.1 Quantification of rpS6 and hsp90 expression patterns…………………101

5.2 The effect of dshsp90 injection into nondiapausing females…………103

A.1 qPCR results showing rpS13 and rpS6 transcriptional levels after low temperature exposure………………………………………………………….116

A.2 qPCR results of rpS13 expression patterns on day 7 and 10 in nondiapausing females after low temperature exposure……………………………..117

A.3 qPCR results showing rpS13 transcriptional levels after exposure at 4°C..118
Chapter 1: Introduction

The *Culex pipiens* mosquito complex is of considerable importance to general and veterinary medicine (Vinogradova, 2000). *C. pipiens* is one of the main vectors of West Nile virus (WNV), an arthropod-borne virus that appeared for the first time in North America in 1999 in the New York City metropolitan area (Centers for Disease Control and Prevention, 2006). By 2003, WNV activity was reported from 46 states with over 9,800 cases of human illness (U.S. Geological Survey, 2009). WNV has been recovered from overwintering *C. pipiens* mosquitoes in New York (Nasci et al., 2001), Pennsylvania (Bugbee and Forte, 2004), and New Jersey (Farajollahi et al., 2005), thus suggesting that the overwintering mosquitoes may be critical for carrying the virus through the winter. WNV is vertically transmitted from mother to offspring during the summer in adult females and stays within the mosquito population until the new cycle begins the following season (Dohm et al., 2002).
Adult diapause in *C. pipiens*

When subjected to harsh environment conditions such as extreme low or high temperature, drought, or lack of food, many insects are able to enter a physiological dormant period known as diapause (Denlinger, 1985). The *C. pipiens* complex in the United State is divided into two major species, the northern house mosquito, *C. pipiens* Linnaeus (previously, *C. pipiens pipiens*) which is found north of 39°N, and the southern house mosquito, *C. quinquefasciatus* Say found south of 39°N (previously, *C. pipiens quinquefasciatus*) (Barr, 1957; Mattingly et al., 1951).

*C. pipiens* has two variants: an autogenous form that can produce eggs without taking a blood meal, and an anautogenous form that is required to take a blood meal to lay eggs and is able to enter diapause (Rouband, 1929; Spielman, 1971; Spielman and Wong, 1973). Only the females have the capacity for diapause. Mating occurs in the fall, and males do not survive the winter. *C. quinquefasciatus* has only an autogenous form and is not able to enter diapause (Vinogradova, 2000).

Diapause is commonly programmed by environmental tokens, e.g. daylength and temperature, that occur well in advance of the diapause stage (Denlinger, 2002). During diapause, development is usually arrested by suppressed/blocked metabolic pathways, and several genes and hormones show unique expression (Denlinger, 1985). During adult diapause in *C. pipiens*, reproduction is suppressed, a common
feature in all adult diapauses; this suppression is sometimes referred to as
‘reproductive diapause’ or ‘gonotrophic dissociation’ which is defined by the failure
of egg maturation even if the female mosquito takes a blood meal (Denlinger, 1985;
Swellengrebel, 1929). Diapause shows various biochemical, physiological, and
behavioral changes, and it is controlled by environmental, hormonal, and molecular
factors. Each level of regulation will be discussed in more detail below.

Environmental regulation of diapause in C. pipiens

Photoperiod and temperature are the main environmental cues controlling
diapause in C. pipiens, as in many other insects. Diapause is programmed in females
between the late 4th instar and early pupa by short day length, e.g. less than 12 hours
day length per day, and low temperature, e.g. below 20°C (Eldridge, 1966; Sanburg
and Larsen, 1973). Diapause-destined female mosquitoes seek hibernation sites
which are relatively warm (0°C ~ 10°C), moist (R.H. > 60%), and dim (< 2-5.2lx),
e.g. caves, culverts or basements, in the late summer and early autumn.

The environmental factors regulating diapause termination are still unclear
in C. pipiens, but previous studies have shown long photophase and/or high
temperature contribute to the decision to terminate diapause (Vinogradova, 2000). In
the case of a closely related species, C. tarsalis, in Boulder Co., Colorado, the
increase in day length is a key factor for diapause termination (Mitchell, 1981; Reisen et al., 1995). Onyeka and Boreham (1987) showed that long day-length exposure is required to break diapause in field-collected *C. pipiens*. By contrast, Oktyabrskaya et al. (1965) reported that the termination of diapause is dependent mainly on temperature in the Moscow area.

*Characteristics of adult diapause in C. pipiens*

When female mosquitoes of *C. pipiens* are preparing to enter diapause, they show several unique characteristics: the absence of host-seeking behavior (Mitchell, 1983; Bowen et al., 1988) and the accumulation of fat reserves (Mitchell and Briegel, 1989). Also, in early diapause, the primary follicles are arrested in a developmental stage classified as Christophers’ Stage I (length is 40 - 50µm) (Christophers, 1911; Spielman and Wong, 1973b). Primary follicles of diapausing mosquitoes in laboratory colonies remain at this stage for 10 weeks and gradually develop to Stage II (70 - 90µm) by 22 weeks (Reisen et al., 1995).

*Hormonal regulation of diapause in C. pipiens*

As in the case of most adult diapauses, juvenile hormone (JH) plays a role as an important negative regulator of diapause in *C. pipiens* (Denlinger, 1985; Spielman,
1974). The amount of JH in the corpora allata, the endocrine gland that produces JH, is four times higher in nondiapausing adult females than in early diapausing females of the same age (Readio et al., 1999). Lack of JH results in a failure of egg maturation, degeneration of flight muscles, and absence of host-seeking behavior (Denlinger, 2002). A role for JH during diapause in C. pipiens is supported by the report that the synthesis of JH triggers termination of diapause (Readio et al., 1999). Also, in other insects, when JH is applied to diapausing adults, diapause is terminated (Denlinger, 1985; 2002).

Molecular regulation of diapause in C. pipiens

Molecular regulation of diapause in C. pipiens remains in an early stage, but our laboratory has been investigating this field with successful progress. Using suppressive subtractive hybridization (SSH), Robich et al. (2007) identified 40 genes that are up-regulated or down-regulated during diapause. Since SSH clones were relatively short, some of the genes of interest required more attention. Robich and Denlinger (2005) showed that three food utilization genes are involved in adult diapause in C. pipiens: trypsin and chymotrypsin-like serine protease, genes that encode blood digestion enzymes, are down-regulated, and fatty acid synthase, a gene encoding an enzyme involving in conversion of sugars to lipid reserves, is up-
regulated in early diapause. Also, insulin signaling, including a downstream molecule, forkhead transcription factor, participates in controlling diapause in this species (Sim and Denlinger, 2008). Moreover, insulin signaling appears to be an upstream pathway regulating JH synthesis in *C. pipiens* (Sim and Denlinger, 2008; 2009). Previously, I reported that two *actin* genes are up-regulated during diapause and redistributed after low temperature exposure, even more so in diapausing mosquitoes (Kim et al., 2006).

**Research foci**

My studies focus on the expression patterns of several genes that have unique expression patterns in association with diapause in *C. pipiens*. Based on previous SSH data generated in our laboratory (Robich et al., 2007), several genes of interest were selected for study. One category of interest included cytoskeletal genes: I previously worked on *actin* (Kim et al., 2006), and in this thesis I examine another cytoskeletal gene, *beta-tubulin*, in relation to diapause and low temperature exposure. Different body regions are examined, and changes of microtubule abundance are also confirmed. The other group selected for study includes *ribosomal proteins*. Several *ribosomal proteins* (*rp*) were investigated for their secondary functions. *RpS3a* and *rpS2* were studied due to their potential roles in ovarian development during
diapause. *Rps6* is examined in correlation with *heat shock protein 90*, and cold-induced *rpS13* is studied in both nondiapausing and diapausing mosquitoes.

Overall, this study investigates cytoskeletal and ribosomal genes that have unique profiles in relation to diapause and low temperature and contribute to expression of the reproductive diapause in *C. pipiens*. 
References


Chapter 2: Decrease in expression of *beta-tubulin* and microtubule abundance in flight muscles during diapause in adults of *Culex pipiens*

Abstract

The cDNA encoding *beta-tubulin* in the mosquito *Culex pipiens* has high similarity with the *beta-tubulins* reported in other insects. In this study, we examine expression of this gene and microtubule abundance in relation to diapause and low temperature. While nondiapausing mosquitoes express *beta-tubulin* highly in their thoracic muscles, expression is quite low during adult diapause. The abundance of microtubules was also much lower in flight muscles of diapausing adults than in flight muscles from nondiapausing individuals, as confirmed by laser confocal microscopy of tubulins stained using indirect immunofluorescence. Low temperature decreased microtubule abundance in midguts of nondiapausing mosquitoes, but microtubule abundance in diapausing mosquitoes was already low and remained unchanged by low temperature exposure. Overall, pixel intensity averages were higher in the flight muscles than in the midguts, and again low temperature decreased microtubule abundance in the flight muscles of nondiapausing females,
while levels remained consistently low in diapausing females. These results clearly indicate that a decrease in microtubule abundance is evoked both by the programming of diapause and, in nondiapausing females, by exposure to low temperature. Quite possibly the reduced microtubule abundance in the flight muscles and reduced expression of \textit{beta-tubulin} are functionally correlated to the reduction in flight activity that is associated with low temperature and diapause.

Introduction

Microtubules, polymers of $\alpha$- and $\beta$-tubulin, are one of the main components of the cytoskeleton and are involved in a range of cellular processes including mitosis, cell motility, cell movement, and cell support (Nogales, 2001). Microtubules, which assemble and disassemble by repeated polymerization and depolymerization of tubulin dimers (Downing and Nogales, 1998), are responsive to temperature changes in a number of organisms, but little information is available for insects. In plants, microtubules are depolymerized in response to low temperature and cause alterations in cell growth (Pihakaski-Maunsbach and Puhakainen, 1995; Nyporko et al., 2003), and in some cases the depolymerization itself acts as a trigger for the cellular response to low temperature (Durso and Cyr, 1994; Mazars et al., 1997). In \textit{Euplotes focardii}, a cold-adapted Antarctic ciliate, depolymerization and
disassembly of microtubules occur at low temperature and are thought to thereby enhance cold tolerance (Pucciarelli et al., 1997; Pucciarelli and Miceli, 2002). In other cases, the opposite response, microtubule assembly, is induced by low temperature, as documented in an Antarctic fish (Detrich et al., 1989; Wallin et al., 1993), fungi (Gupta et al., 2001), and algae (Willem et al., 1999).

The relationship between diapause and cold tolerance has been studied in several insects including the flesh fly, *Sarcophaga crassipalpis*, that diapauses as a pupa (Adedokun and Denlinger, 1984), the Colorado potato beetle, *Leptinotarsa decemlineata*, that diapauses as an adult (Lefevere et al., 1989), and the mosquito, *Aedes albopictus*, that diapauses as a pharate first instar (Hanson and Craig, 1994). In all of these cases both diapause and low temperature increase cold tolerance.

*Culex pipiens* is also a species in which cold tolerance is a component of the diapause program: diapausing females survive 5-10 times longer than nondiapausing females when exposed to certain low temperature regimes (Rinehart et al., 2006). The overwintering diapause of *C. pipiens*, and the enhanced cold tolerance that is part of the diapause syndrome, are programmed by the short days of early autumn (Sanburg and Larsen, 1973).

Only a few studies in insects have reported cytoskeletal responses to low temperature and/or diapause. In pharate larvae of the gypsy moth, *Lymantria dispar*,
a brain-specific actin is down-regulated during diapause (Lee et al., 1998). In the mosquito, *C. pipiens*, two actin genes respond to low temperature and are highly expressed early in adult diapause. Changes in these genes were accompanied by a conspicuous redistribution of polymerized actin that was most pronounced in the midguts of diapausing mosquitoes exposed to low temperature (Kim et al., 2006). Expression of the gene encoding *beta-tubulin* was reported to be unchanged in the whole body during adult diapause in *C. pipiens* (Robich et al., 2007), but in the present study, we examine expression of *beta-tubulin* in different body regions and report down-regulation of this gene in the flight muscles during diapause. We confirm distribution of the protein using laser confocal microscopy and report a decrease in microtubules in the flight muscles during diapause, and, finally, we report the responsiveness of microtubules to low temperature.

**Materials and Methods**

*Insect rearing*

The colony of *Culex pipiens pipiens* (Buckeye strain) was maintained at 25°C, 75% R.H., with a 15L (light):9D (dark) cycle (Nondiapause, 25°C), as previously described (Robich and Denlinger, 2005). Diapause was programmed by moving second instar larvae to an environmental room at 18°C, 75% R.H., with a
9L:15D cycle (Diapause, 18°C). As a control, some second instar larvae were allowed to develop in an environmental chamber at 18°C, 75% R.H., with a 15L:9D cycle (Nondiapause, 18°C).

Full-length sequence

Total RNA was extracted from females using TRIZOL Reagent (GIBCO BRL, USA) following the manufacturer’s instructions. Absorbance was measured to quantify RNA by a spectrophotometer at 260nm (BioSpecmini, Shimadzu). The 3’ Rapid Amplification of cDNA Ends kit (Invitrogen) was used to obtain full length sequence of beta-tubulin according to the manufacturer’s instructions. The initial partial sequence of beta-tubulin (241bp) was obtained by Robich et al. (2007). Amplification of the target cDNA was performed twice until the full length sequence was obtained. The forward primers were 5’-GAT GCG CTT GAA CAG CTC CT-3’ (first amplification), and 5’-ACT GGG CCA AGG GAC ACT AC-3’ (second amplification), and the Abridged Universal Amplification Primer (AUAP) provided within the kit as the reverse primer was 5’-GGC CAC GCG TCG ACT AGT AC-3’. The final PCR product was sequenced at the Plant-Microbe Genomics Facility at The Ohio State University using an Applied Biosystem 3730 DNA Analyzer and BigDye™ cycle sequencing terminator chemistry.
Northern blot hybridization

RNAs were separately extracted from the head, thorax, and abdomen in nondiapausing females reared at 25°C, nondiapausing females reared at 18°C, and diapausing females reared at 18°C. Early (7-10 days after adult eclosion) female mosquitoes were used to compare nondiapausing (reared at 25°C and 18°C) and diapausing (reared at 18°C) groups. In addition, early and mid (1 month after adult eclosion) diapause mosquitoes were compared. Formaldehyde denaturing RNA gels were transferred onto Hybond-nylon positive membrane (Amersham Bioscience) by Schleicher and Schuell TurboBlotter system. Digoxigenin (DIG)-labeled beta-tubulin cDNA was prepared by RT-PCR, and a beta-tubulin probe was labeled using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences). To verify equal quantity of loaded RNAs, DIG-labeled 28S cDNA was used. All Northern blot hybridization results were replicated three times.

Indirect fluorescence staining and microscopy

To confirm beta-tubulin abundance in different body regions, the thoracic flight muscles and the midguts (abdomen) were dissected, fixed in 2% paraformaldehyde in PBS (phosphate buffered saline), and incubated in 0.1% Triton X-100 in PBS for 5 min and 1% bovine serum albumin (BSA) for 15 min at room
temperature to minimize nonspecific background staining. An indirect immunofluorescence method was used to prevent inhibition of microtubule assembly and poly/depolymerization in tissues and to increase the microtubule-specificity of antibodies (Blose et al., 1984; Breitling and Little, 1986). Monoclonal anti-β-tubulin antibody was produced in mouse (SIGMA, T4026); the primary antibody was diluted 1:1000 using 1% BSA in PBS for 1 h at 37°C. The anti-Mouse IgG-FITC antibody was produced in goat (SIGMA, F5262); the secondary fluorescent antibody was diluted 1:300 using 1% BSA in PBS for 1 h at 37°C. Tissues were washed three times in PBS after each step, except after the incubation of Triton X-100 solution. Samples were air dried, mounted on slides in a mountant (Cytoseal™), and examined within 24 h on a Zeiss 510 META laser scanning confocal microscope at the Campus Microscope and Imaging Facility (CMIF) at The Ohio State University.

Cold stress at low temperature

Nondiapause (reared at 25°C and 18°C) and diapausing (reared at 18°C) female mosquitoes one month after adult eclosion were exposed to 0°C or -5°C for 12 h using a water bath contained water and glycerol (50:50). Mosquitoes were chilled at -70°C for 20 sec and immediately dissected in insect saline (Ringer, 1883) under a stereo microscope (Wild Heerbrugg M8) and immediately placed on ice.
Eight females were cold-treated and dissected for each treatment, and all treatments were repeated three times. Dissected tissues were stained and examined under a microscope as described above.

Bioinformatics and Statistics

To compare a multiple sequence alignment of beta-tubulin in other species, BLASTp in GenBank (http://www.ncbi.nlm.nih.gov/), Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) were used. ImageJ (http://rsb.info.nih.gov/ij) was used for resizing and calculating mean pixel intensity, which was used to compare tubulin abundance. Graphs, the Mann-Whitney test, and two way ANOVA were obtained using MiniTab (version14).

Results

Beta-tubulin sequence

The full-length cDNA of beta-tubulin is 1395bp and encodes a protein of 443 amino acids (GenBank accession number DQ401465; ABG29421). The open reading frame (ORF) of this gene is 1332bp, from nucleotides 1 to 1332, with a 63bp 3’ untranslated region including the poly-A tail. The putative polyadenylation signal
(AATAAA) is found at nucleotides 1337-1342. A multiple sequence alignment of the beta-tubulin amino acid sequence with the sequences of three other insect beta-tubulins is shown in Figure 1. The ORF of beta-tubulin from *C. pipiens pipiens* has 99% similarity at the amino acid level with the ORF from another mosquito species, *Aedes aegypti*, and also with the fruit fly, *Drosophila melanogaster*. The beta-tubulin amino acid sequence from *C. pipiens* also shares 98% similarity with that from the silk moth, *Bombyx mori*.

*Low beta-tubulin expression in the thorax during diapause*

Three different body regions, head, thorax, and abdomen, were compared in nondiapausing (reared at 25°C and 18°C in long-day length) and diapausing (reared at 18°C in short-day length) females of *C. pipiens*. Figure 2 shows expression levels in the three body regions 7-10 days after adult eclosion in the two types of mosquitoes. Abdomens of diapausing (18°C) and nondiapausing (18°C and 25°C) mosquitoes expressed the beta-tubulin gene at similar levels, while expression of beta-tubulin in the thoraces of diapausing mosquitoes was much less than in the thoraces of nondiapausing mosquitoes. Expression in the heads of diapausing (18°C) mosquitoes was slightly less than in the heads of nondiapausing mosquitoes (both at 18°C and 25°C). We also checked older diapausing females (1 month after adult
eclosion), and they displayed the same expression pattern observed in the early (7-10
days after adult eclosion) diapausing females (Figure 2). The thorax is mostly
composed of flight muscles. Thus, we assume this observed difference reflects a
down-regulation of beta-tubulin within that tissue during diapause.

*Microtubule abundance monitored by histochemistry*

Microtubule abundance in different body regions was evaluated using laser
scanning confocal microscopy. Figure 3 A shows images of the midgut with
microtubule staining in early nondiapausing and diapausing mosquitoes reared at 18°C.
Nondiapausing and diapausing images showed similar structural patterns of
tubulin expression in the midguts, and statistical analysis indicated no significant
differences in the midguts of diapausing and nondiapausing mosquitoes reared at 18°C
(Figure 3 B). Both the pattern and abundance of tubulin remained identical in the
two groups. Microtubule structure was also evaluated in thoracic muscles in early
nondiapausing and diapausing mosquitoes (Figure 4 A). Although the thoracic
muscle images did not show obvious differences in tubulin structure, statistical
analysis of the images from early nondiapausing and diapausing mosquitoes
indicated significantly less staining of tubulins in the diapausing mosquitoes (Figure
4 B). These results are consistent with the Northern blot hybridization results shown
in Figure 2, which indicate thorax-specific differences in *beta-tubulin* gene expression linked to diapause.

*Changes in microtubules elicited by cold*

Microtubule abundance was also evaluated in nondiapausing and diapausing mosquitoes in response to low temperature. An analysis of pixel intensity indicates that the tubulin level dramatically decreased after exposure to 0°C and even further after exposure to -5°C in midguts of nondiapausing mosquitoes reared at 25 ºC. However, the tubulin level in diapausing mosquitoes reared at 18 ºC was unchanged following exposure to low temperatures of 0°C and -5°C, and remained consistently at the low level observed for nondiapausing mosquitoes held at -5°C. Average pixel intensity was higher in thoracic muscles than in midguts, and the thoracic muscles of nondiapausing mosquitoes reared at either 25°C or 18°C responded to low temperatures by decreasing tubulin abundance (Figure 5). The fact that a drop in temperature caused a major decline of tubulin in both the midgut and flight muscle of nondiapausing mosquitoes suggests an important role for temperature in the amount of tubulin present. It is also evident that the amount of tubulin was consistently low in tissues from diapausing adults (reared at 18ºC). Results of nondiapausing mosquitoes reared at 18ºC (middle parts in Figure 5) indicate an
intermediate response. Clearly temperature by itself can alter the abundance of tubulin, but the programming of diapause, regardless of rearing temperature, elicits a shift toward low microtubule abundance in the flight muscles.

Discussion

Tubulin, a globular protein that is the main constituent of microtubules, is present as a cytoskeletal component of most eukaryotic cells and serves diverse functional roles (McKean et al., 2001). In this study we obtained a full-length cDNA (1395bp) of the gene that encodes this protein in C. pipiens. A previous study in C. pipiens did not show changes of beta-tubulin during adult diapause, but that conclusion was based on whole body assays (Robich et al., 2007). In the present study, we examined gene expression in different body regions (head, thorax, and abdomen) and noted different expression patterns in the thoraces of diapausing and nondiapausing mosquitoes. Even though tubulins are present in the most tissues, they are known to have tissue-specific expression and specialized functions (Theurkauf et al., 1986; Carpenter et al., 1992; Kawasaki et al., 2003). In our study, beta-tubulin expression was significantly lower in the thorax of diapausing mosquitoes than in nondiapausing mosquitoes, differences in levels of expression were less pronounced in the head, and not at all evident in the abdomen. These distinct patterns of
expression were noted during both early and mid diapause. Laser scanning confocal microscopy data using an indirect fluorescence staining method to detect tubulin also supported these results.

We suspect that the low expression of \textit{beta-tubulin} and lower abundance of tubulin in the flight muscles is a reflection of the low level of flight activity during diapause or alternatively may represent an adaptation that enhances function at low temperature. Diapausing adults of \textit{C. pipiens} are indeed able to fly (Buffington, 1972), but they are not as active as nondiapauing females because they are not engaged in host-seeking behavior, and they fly very little during the overwintering months (Mitchell, 1983; Bowen et al., 1988). Flight muscle degeneration has been studied during diapause in several other insects. In the Colorado potato beetle, \textit{L. decemlineata}, flight muscles completely degenerate during diapause (Stegwee et al., 1963), a condition that is also observed during diapause in a tropical beetle, \textit{Stenotarsus rotundus} (Wolda and Denlinger, 1984). Flight muscles are expensive to maintain, and the degeneration of flight muscles during diapause is assumed to reduce energy demands during this period (Denlinger, 1986). But, unlike these beetle examples, \textit{C. pipiens} retains use of its flight muscles during diapause and is fully capable of flying. Yet, clearly there are some alterations of the flight muscles during the diapause of \textit{C. pipiens}, as reflected in our observation in this study.
We also showed that low temperature, as well as diapause, can influence the amount of tubulin present. Low temperature dramatically reduced microtubule abundance in both the midgut and thoracic muscles of nondiapausing mosquitoes. By contrast, low microtubule abundance was consistently observed in diapausing mosquitoes, regardless of temperature. This suggests that the assembly and disassembly of microtubules in C. pipiens can be influenced both by diapause and temperature, and that microtubules (tubulin), as well as actin filaments (Kim et al., 2006), are involved in diapause and the cold response of this species.

Although an alteration in microtubules is a common response to low temperature it is a bit strange that in many cases this involves microtubule assembly, while in a few cases low temperature evokes microtubule disassembly, as cited in the Introduction. Disassembly of the microtubules was evident in this study of C. pipiens, a response much akin to the low temperature response noted for the Antarctic ciliate, E. focardii (Pucciarelli and Miceli, 2002). Disassembly involves depolymerization of the microtubules, a process initiated by GTP hydrolysis (Mitchison and Kirschner, 1984; Erickson and O’Brien, 1992; Bayley et al., 1994) and enhanced by calcium ions (O’Brien et al., 1997). Calcium signaling appears to mediate low temperature responses of plants (Bush, 1995; Monroy et al., 1993; Monroy and Dhindsa, 1995), and there is recent evidence that calcium also functions as a second messenger
mediating the low temperature response of at least one insect, the Antarctic midge

_Belgica antarctica_ (Teets et al., 2008). Though the signal transduction pathway for
low temperature has not yet been determined for _C. pipiens_, it is evident that both
diapause and low temperature evoke a fundamental change in tubulin formation, and
as in other systems, calcium would be a likely candidate as a signaling molecule to
trigger such changes.
References


Ringer, S. (1883) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. The Journal of Physiology 4: 29-42.


Figure 2.1. Multiple sequence alignment of beta-tubulin from Culex pipiens and 3 other insects: Cuxpi: C. pipiens pipiens beta-tublin ABG29421; Aedae: Aedes aegypti beta-tublin chain EAT45945; Dromo: Drosophila melanogaster beta-tublin isoform B NP523795; Bommo: Bombyx mori beta-tublin NP001036964.
Figure 2.2. Northern blot hybridization analysis of *beta-tubulin* expression 7-10 days after adult eclosion in nondiapausing mosquitoes reared at 25°C (ND25), nondiapausing mosquitoes reared at 18°C (ND18), diapausing mosquitoes reared at 18°C (Early D18), and 1 month after adult eclosion in diapausing mosquitoes reared at 18°C (Mid D18). H, T, and A represent head, thorax, and abdomen, respectively. One representative replicate is shown; all three replicates showed nearly identical expression patterns.
Figure 2.3. (A) Laser scanning confocal microscopy images of midgut tubulin in early nondiapausing mosquitoes reared at 18°C (ND18) and diapausing mosquitoes reared at 18°C (D18). White scale bar in left bottom indicates 25μm. (B) The Mann-Whitney test: Interval plots of pixel intensity of tubulin-stained midgut images in early ND18 and D18 showing no significant differences between the two groups. Each interval plot was analyzed using 4 midgut images in each condition. The dot in the line marks the mean for each condition and the line shows the mean ± 95% confidence interval. P-Value is 0.471.
Figure 2.4. (A) Laser scanning confocal microscopy images of tubulin in thoracic muscles from early nondiapausing mosquitoes reared at 18°C (ND18) and diapausing mosquitoes reared at 18°C (D18). White scale bar in left bottom indicates 25um. (B) The Mann-Whitney test: Interval plots of pixel intensity of tubulin-stained thoracic muscle images in early ND18 and D18 showing significant differences between the two groups. Each interval plot was analyzed using 8 thoracic muscle images in each condition. The dot in the line marks the mean for each condition and the line shows the mean ± 95% confidence interval. P-Value is 0.003.
Figure 2.5. Interval plots of pixel intensity of tubulin in (A) midgut and (B) thoracic muscle images, 1 month after adult eclosion for nondiapausing mosquitoes reared at 25°C (ND25), nondiapausing mosquitoes reared at 18°C (ND18), and diapausing mosquitoes reared at 18°C (D18). Each plot was analyzed using 8 images. Two way ANOVA of three groups in (A) shows a P-value of 0.022. Two way ANOVA of three groups in (B) shows a P-value of 0.001. 0°C and -5°C represent the tested samples after exposure to 0°C or -5°C for 12 h. 25°C and 18°C represent groups without exposure to low temperature. The dot in the line marks the mean for each condition and the line shows the mean ± 95% confidence interval.
Chapter 3: RNA interference directed against ribosomal protein S3a suggests a link between this gene and arrested ovarian development during adult diapause in *Culex pipiens*

Abstract

Arrested ovarian development is a key characteristic of adult diapause in the mosquito, *Culex pipiens*. In this study we propose that ribosomal protein S3a (rpS3a), a small ribosomal subunit, contributes to this shut down. *RpS3a* is consistently expressed in females of *C. pipiens* that do not enter diapause, but in females programmed for diapause, expression of the *rpS3a* transcript is dramatically reduced for a brief period in early diapause (7-10 days after adult eclosion). RNA interference directed against *rpS3a* in nondiapausing females arrested follicle development, mimicking the diapause state. The effect of the dsRNA injection faded within 10 days, allowing the follicles to again grow, thus the suppression of *rpS3a* caused by RNAi did not permanently block ovarian development, implying that a brief suppression of *rpS3a* is not the only factor contributing to the diapause response. The arrest in development that we observed in dsRNA-injected females could be reversed
with a topical application of juvenile hormone III, an endocrine trigger known to terminate diapause in this species. Though we speculate that many genes contribute to the diapause syndrome in *C. pipiens*, our results suggest that a shut down in the expression of *rpS3a* is one of the important components of this developmental response.

**Introduction**

Adult females of *Culex pipiens* overwinter in diapause, a dormancy that is characterized by the absence of host-seeking behavior (Bowen et al., 1988; Mitchell, 1983), accumulation of huge fat reserves (Mitchell and Briegel, 1989; Robich and Denlinger, 2005), and an arrest of ovarian development (Christophers, 1911; Spielman and Wong, 1973b). This diapause is induced environmentally by short day length and low temperature (Sanburg and Larsen, 1973; Spielman and Wong, 1973a; 1973b), and at the hormonal level by a shut-down in juvenile hormone (JH) production (Spielman, 1974; Readio et al., 1999). During diapause, the insulin pathway is blocked (Sim and Denlinger, 2008; 2009), a response that we suspect leads to a shutdown of JH synthesis, the accumulation of fat reserves and enhanced stress-resistance.
Ribosome biogenesis is closely linked to the regulation of four ribosomal RNAs (rRNA) and 70-80 ribosomal proteins (rp) (Mager, 1988; Niu and Fallon, 1999). These ribosomal proteins are among the major control points regulating mosquito reproduction, as demonstrated in *Aedes aegypti* (Raikhel, 1992). Ribosome synthesis supplies the translational machinery to maintain protein production for vitellogenesis in the mosquito fat body (Hagedorn et al., 1973; Raikhel et al., 2002), but ribosomal proteins have numerous secondary functions as well (Niu and Fallon, 2000; Wool, 1993). For example, ribosomal protein S3a (rpS3a) has numerous physiological functions including roles in apoptosis (Naora et al., 1995; 1998), cell transformation (Lecomte et al., 1997; Naora et al., 1996), and initiation of translation (Tolan et al., 1983). *RpS3a* also plays a role in oogenesis. In *Drosophila melanogaster*, ovarian development is inhibited when *rpS3a* is suppressed with an antisense construct (Reynaud et al., 1997), and the gene is up-regulated during oogenesis in *Anopheles gambiae* (Zurita et al., 1997). Using suppressive subtractive hybridization (SSH) Robich et al. (2007) showed that *rpS3a* is among the genes that are down-regulated during early diapause in *C. pipiens*. In this paper we further evaluate the relationship between *rpS3a* and diapause. We define a narrow temporal window during which this gene is down-regulated during diapause and use RNA interference (RNAi) to suppress *rpS3a* in nondiapausing females. This suppression
results in a developmental arrest that mimics the ovarian arrest characteristic of diapause. We also show that this arrest can be countered with an application of juvenile hormone, the endocrine signal that normally terminates diapause in this species.

Materials and Methods

Insect rearing

The colony of *C. pipiens* (Buckeye strain) was maintained at 25°C, 75% R.H., with a 15L (light):9D (dark) cycle (Nondiapause, 25°C). Ground fish food (TetraMin, Tetra, Blacksburg, VA, USA) was provided as the larval food. As second instar larvae, experimental groups were transferred to either 18°C, 75% R.H., 9L:15D to program diapause (Diapause, 18°C) or to 18°C, 75% R.H., 15L:9D as a nondiapause control group (Nondiapause, 18°C). Adult mosquitoes were provided water and a 10% sucrose solution. The sucrose solution was removed from the cage of diapause-programmed mosquitoes 10 days after adult eclosion to mimic the low availability of a sugar source during the overwintering period.
**Cloning rpS3a and Northern blot hybridization**

The gene encoding rpS3a was cloned using a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) with forward and reverse primers (5’-CAC GCC TTC TCA ATG TCC TT -3’ and 5’-AAG GTT GTG GAT CCG TTC AC -3’)
designed from the retrieved sequences reported by Robich et al. (2007) and the *A. aegypti* rpS3a sequence from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) using BLAST programs. Sequence of ribosomal protein L19 (*rpL19*), used as a control, was archived in VectorBase (www.vectorbase.org, CPIJ014540-RA) and cloned as described above with primers (forward primer: 5’-ATG AGT TCC CTC AAG CTC CA -3’, reverse primer: 5’-ATC AGG ATG CGC TTG TTC TT -3’).

Total RNA was extracted from female mosquitoes using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.

Absorbance was measured to quantify RNA by a spectrophotometer at 260nm (BioSpecmini, Shimadzu, Kyoto, Japan). For ovarian RNAs, 50 ovaries from 25 females were collected in each condition. Formaldehyde denaturing RNA gels were transferred onto a Hybond-nylon positive membrane (GE Healthcare Bio-Science, Uppsala, Sweden) using a Schleicher and Schuell TurboBlotter system (Schleicher and Schuell BioScience Inc, Keene, NH, USA). The *rpS3a* and *rpL19* probes were
labeled using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences, Indianapolis, IN, USA). 28S ribosomal RNA from *C. pipiens* was used to verify equal loading. All Northern blot hybridization results were replicated three times.

dsRNA of *rpS3a*

dsRNA of *rpS3a* using T7 (5’-TAA TAC GAC TCA CTA TAG GG -3’) - forward primer (5’-CGT TGT TGG TGA TGA TCT GG -3’) and T7-reverse primer (5’-AAG GTT GTG GAT CCG TTC AC -3’), dsRNA of *rpL19* using T7-primers (forward primer: 5’-ATG AGT TCC CTC AAG CTC CA -3’, reverse primer: 5’-ATC AGG ATG CGC TTG TTC TT -3’), and dsRNA of β-galactosidase used as the second control (primers described in Sim and Denlinger, 2009) were prepared by the MEGAscript T7 transcription kit (Ambion, Applied Biosystems, Foster City, CA, USA) as previously described (Sim et al., 2007). 0.4µg/µl dsrpS3a or 0.5µg/µl dsrpL19 or 0.5µg/µl dsβ-gal was injected into the thorax of cold, carbon dioxide-anesthetized nondiapausing female mosquitoes (reared at 18°C) 4 days after eclosion using a microinjector (Tritech Research, Los Angeles, CA, USA). All injected nondiapausing mosquitoes were placed at 18°C, 75% R.H., 15L:9D with cotton wicks soaked in a 10% sucrose solution. Ovaries of dsRNA injected mosquitoes
(rpS3a, rpL19 and β-gal) were dissected 2, 4, 10, 20, and 30 days after injection, and the length of the primary follicles were measured using an Inverted Tissue Culture Microscope (Nikon Diaphot, Melville, New York, USA). To confirm RNAi efficiency, Northern blot hybridization was performed using RNAs extracted from dsrpS3a and dsrpL19 injected mosquitoes. Follicles were separated from ovaries using #00 insect pins (0.3mm diameter) under a microscope, and images of each follicle were made using a Zeiss Axioskop Widefield Light Microscope at the Campus Microscopy and Imaging Facility at The Ohio State University.

**JHIII application**

Juvenile hormone (JH) III (Sigma-Aldrich, St. Louis, MO, USA) was used in experiments designed to rescue the developmental arrest caused by an injection of dsrpS3a. 0.1µg/µl JHIII diluted in acetone was topically applied to females immediately after injection of dsrpS3a. Lengths of primary follicles were measured 2 and 4 days after injection/application as described above.

**Statistics**

One-way ANOVA using MINITAB (Minitab Inc., version 14, State College, PA, USA) was used to analyze the statistical significance of differences in length of
the primary follicles. A pair-wise comparison was used for multiple treatment comparisons, and a 5% significance level was applied to all tests.

Results

Low expression of rpS3a during early diapause

Northern blot hybridization showed that the rpS3a transcript was continuously expressed in pupae and in nondiapausing female adults for at least 2 months after adult eclosion (Figure 1 A), but in diapausing mosquitoes, expression of the transcript was low in females collected 7-10 days after eclosion (Figure 1 B). Additional samples collected 8 and 10 days after adult eclosion confirmed low expression of the transcript in diapausing females during this period (Figure 1 C).

These results differ slightly from results reported in a previous paper from our laboratory (Robich et al., 2007). Both studies showed low expression of rpS3a mRNA in early diapause and high expression in late diapause, but the earlier study did not show evidence of rpS3a expression in the nondiapausing sample. We attribute this difference to the fact that the earlier study was based on a much smaller fragment (469bp clone) than used in this study (1200bp clone).

In addition to the whole body results presented in Figure 1, we examined expression in the ovaries and confirmed that the down-regulation of rpS3a was also
Arrested ovarian development following dsRNA of rpS3a in nondiapausing females

RNAi was used to directly evaluate the function of rpS3a. An injection of dsrpS3a into nondiapausing mosquitoes suppressed ovarian development and mimicked the arrest observed in diapause. Follicles observed 2 days after dsrpS3a injection into 4 day-old nondiapause mosquitoes were significantly smaller than those of 6 day-old nondiapause mosquitoes (Figure 3 A, P = 0.001, One-way ANOVA). Follicles were still in a stage of arrest 4 days after the dsRNA injection; the arrested follicles of the dsRNA-injected nondiapause mosquitoes were similar in size and shape to follicles dissected from diapausing mosquitoes (Figure 3 A and C). The arrest we noted using dsrpS3a was not seen in either the dsrpL19 (another ribosomal protein) or dsβ-gal controls (Figure 3 A). Northern blot hybridization confirmed a reduction in the level of transcript following RNAi for rpS3a and rpL19 (Figure 3 B). Pair-wise comparison showed significant differences between the nondiapause control group and the dsrpS3a-injected group, and between the dsrpS3a-injected group and the dsrpL19-injected group (P < 0.01) at the 5% significance level. These results suggest that rpS3a is critical for the progression of ovarian development in C. pipiens.
To confirm that RNAi directed against \( rpS3a \) suppressed expression not only in the whole body but also within the ovary, we used Northern blot hybridization to evaluate expression within the ovary. As shown in Figure 2 B, an injection of dsrpS3a into nondiapausing females did indeed suppress expression of \( rpS3a \) within the ovary.

**RNAi effect degrades with time**

Duration of the RNAi effect was tracked by measuring primary follicle length at different intervals after dsRNA injection (Figure 4). Although the follicles were clearly arrested 2 and 4 days after dsRNA injection, development was again evident 10 days after injection. Follicle length gradually increased with time (Figure 4). When measured 20 and 30 days after injection, follicle lengths of dsRNA injected females were nearly identical to that of non-injected nondiapausing controls.

**JHIII rescues the arrested ovarian development**

JHIII was used in an attempt to rescue the arrest in ovarian development caused by dsRNA of \( rpS3a \). Primary follicles were significantly larger when dsRNA-injected females received an application of JHIII: this effect was already evident 2 days after JHIII treatment and was quite pronounced by 4 days (Figure 5). Pair-wise
comparisons showed that the only significant differences at the 5% level were between the nondiapause and diapause controls and between the nondiapause controls and those injected with dsrpS3a. These results clearly show that JHIII can rescue the \textit{rpS3a} suppressed mosquitoes, thus suggesting a causative link between suppression of \textit{rpS3a}, ovarian developmental arrest and the JH deficiency known to regulate adult diapause in this mosquito.

Discussion

The evidence we present in this study is consistent with the idea that \textit{rpS3a} may be a link in the diapause mechanism. Although \textit{rpS3a} is highly expressed in both nondiapausing and diapausing females at the very beginning of adult life and also at an age of one month, the gene is down-regulated for a brief period, 7-10 days after adult eclosion, in diapausing females. This down-regulation is evident not only in whole bodies but also within the ovaries. We used RNAi directed against \textit{rpS3a} to see if we could mimic a “diapause-like” ovarian arrest in nondiapausing females, and indeed dsrpS3a successfully arrested development in these females. Diapause, of course, represents more than a single gene response, and the arrest we observed was not a long-term arrest that is fully equivalent to diapause. Also the fact that the RNAi-induced suppression in nondiapausing females was not as pronounced as the
naturally-occurring down-regulation of \(rpS3a\) during diapause likely contributes to the abbreviated arrest we observed. We suspect that the precise timing of \(rpS3a\) suppression is also critically important. But, taken together our current results suggest that the down-regulation of \(rpS3a\) noted in diapause-destined mosquito females is likely to be an important component of the diapause syndrome.

The duration of dsRNA in a test organism is critical for the success of an RNAi experiment (Montgomery et al., 1998). The gene knockdown effects of dsRNA are frequently maintained for 3-10 days, depending on the gene and organism (Byrom et al., 2002; Dorn et al., 2004; Hemmings-Mieszczak et al., 2003). In our experiments, the RNAi effect remained for at least 4 days but was lost after 10 days.

In diapausing females of \(C. pipiens\), the arrest in ovarian development is prompted by a shut-down in the production of JH (Readio et al., 1999), and the arrest can be terminated by application of exogenous JH (Spielman, 1974). We have shown that JHIII, the juvenile hormone found in mosquitoes (Zhu et al., 2003), is also capable of terminating the arrest in ovarian development caused by suppression of \(rpS3a\). Together, these results are consistent with the hypothesis that the arrest in ovarian development that characterizes diapause of \(C. pipiens\) is linked to reduced expression of \(rpS3a\). It is not yet clear how JH rescues the arrested development.
One might assume that the naturally high JH titer present in nondiapausing females would be sufficient to counter the dsRNA-induced arrest, but this apparently is not the case. Possibly suppression of rpS3a is somehow directly or indirectly blocking JH production or action, but these scenarios remain to be tested.

Although ribosomal proteins are best known for their primary role in protein synthesis (Wool et al., 1995; Wool, 1996), it is clear that many ribosomal proteins also serve secondary functions that differ substantially from their role in regulating protein synthesis (Coetzee et al., 1994; Hart et al., 1993; Kim et al., 2004; Watson et al., 1992; Wu et al., 2008). Specifically, down-regulation of rpS3a has been linked in several cases to an arrest in growth, e.g. in erythrocytes (Cui et al., 2000) and NIH 3T3 cells (Naora et al., 1998). In insects, one of the reported functions of rpS3a is a role in oogenesis. The rpS3a gene in D. melanogaster is highly expressed in follicular epithelial cells of the ovary (Reynaud et al., 1997), and using transgenic flies, they confirmed that suppression of the rpS3a gene causes an abnormality of oogenesis: egg production was significantly decreased in an antisense transgenic line. In the mosquito, A. gambiae, this gene is up-regulated in the ovary during oogenesis (Zurita et al., 1997). Our data on C. pipiens also supports a role for rpS3a in oogenesis, and we suggest that suppression of this gene is a feature of reproductive diapause in this species. Whether the role of rpS3a in diapause is strictly related to
protein synthesis or may involve a secondary role is unresolved, but it is clear that there is some specificity to this response, as demonstrated by the fact that suppression of *rpS3a* elicits the diapause-like arrest, while suppression of another ribosomal protein, *rpL19*, does not.
References


specific inhibition of the recombinant Rat P2X3 receptor. Nucleic Acids Research 31:2117-2126.


Figure 3.1. Northern blot hybridization showing expression of the mRNA encoding ribosomal protein S3a (rpS3a). (A) *RpS3a* was continuously expressed in nondiapausing females reared at 18°C long days. (B) In diapause mosquitoes, reared at 18°C short days, *rpS3a* was down-regulated in early (7-10 days) stages of diapause. (C) Further confirmation of the low expression of *rpS3a* on day 8 and 10 diapausing females. 28S ribosomal RNA was used as a control.
Figure 3.2. Northern blot hybridization confirming expression of *rpS3a* in the ovaries. (A) *RpS3a* expression was high in ovaries of nondiapausing mosquitoes (ND) 10 days after eclosion, but down-regulated in diapausing females of the same age. *Rpl19* was used to confirm equal loading. (B) *RpS3a* expression indicates suppressed expression in the ovaries of nondiapausing females injected with dsrpS3a on day 4 and examined on day 6.
Figure 3.3. The effect of injection of dsRNA on expression of $rps3a$. (A) Mean ± SD length of primary follicles after injection of ds$rps3a$ (10 follicles measured per mosquito; total of 10 mosquitoes for controls and 25 mosquitoes for others. ND = nondiapause mosquitoes, D = diapause mosquitoes, d = days after adult eclosion, dai = days after injection). Ovarian development was arrested when $rps3a$ was suppressed by dsRNA injection. dsRNAs of $rpl19$ and $\beta$-gal were injected as internal controls. Bars with the same letters are not significantly different (P > 0.05). (B) Northern blot hybridization confirmed suppression of $rps3a$ (left) and suppression of $rpl19$ (right) in nondiapause mosquitoes. 28S ribosomal RNA was used as a control. (C) Primary follicle images: a. Nondiapause mosquito, 6 days, b.
Nondiapause mosquito, 8 days, c. Diapause mosquito, 8 days, d. Nondiapause mosquito, 4 days + 2 days after injection of dsrpS3a, e. Nondiapause mosquito, 4 days + 4 days after injection of dsrpS3a. (Scale bar = 50μm)
Figure 3.4. The effect of dsrpS3a in nondiapausing mosquitoes at different times after injection. Mean ± SD primary follicle length slightly increased with time after dsrpS3a injection due to the presumed degradation of dsRNA (10 follicles measured per mosquito; total 10 mosquitoes for controls and 25 mosquitoes for others). d = days after adult eclosion, dai = days after injection of dsrpS3a. Bars with the same letters are not significantly different (P > 0.05).
Figure 3.5. Juvenile hormone (JHIII) rescues the ovarian developmental arrest caused by an injection of dsrpS3a. Mean ± SD primary follicles initiated development when JHIII was applied immediately after a dsrpS3a injection (10 follicles measured per mosquito; total 10 mosquitoes for controls and 25 mosquitoes for others). ND = nondiapause mosquitoes, D = diapause mosquitoes, d = days after adult eclosion, dai = days after injection of dsrpS3a. Bars with the same letters are not significantly different (P > 0.05).
Chapter 4: A potential role for ribosomal protein S2 in the gene network regulating reproductive diapause in the mosquito *Culex pipiens*

Abstract

A shut-down in expression of ribosomal protein S2 (*rpS2*) appears to contribute to regulation of diapause in adult females of *Culex pipiens*. While this gene is expressed continuously in nondiapausing females reared under long-day conditions, it is strongly down-regulated 5-18 days after adult eclosion in diapausing females reared under short-day conditions. The possibility that this shut-down in expression of *rpS2* contributes to the arrest in ovarian development characteristic of diapause is bolstered by the diapause-like arrest in follicle growth observed when nondiapausing females were injected with dsrpS2. A control gene encoding another ribosomal protein, *L19*, is expressed equally in nondiapausing and diapausing females, and RNA interference directed against *rpL19* did not arrest follicle growth, thus indicating that the response we observed in knocking down expression of *rpS2* is not common to all ribosomal proteins. Diapause in *C. pipiens* is readily terminated with juvenile hormone (JH), and in this study we demonstrated that an exogenous
application of JHIII can rescue the arrest in follicle growth caused by dsrpS2.

Together, these results suggest that rpS2 plays a critical role in arresting the ovarian development associated with diapause in this mosquito.

Introduction

Ribosomal proteins (rp) contribute to an array of reproductive processes including oogenesis, vitellogenesis, and embryogenesis (Davidson, 1976; Postlethwait and Giorgi, 1985), but it is also clear that, in addition to their roles in regulating protein synthesis, rps can exert multiple roles on cellular processes (Maquire and Zimmermann, 2001; Sanyal and Liljas, 2000; Wool, 1993). Specific regulatory roles relevant to the control of oogenesis and embryogenesis have been identified in a range of animals including mice (LaMarca and Wassarman, 1979), frogs (Hyman and Wormington, 1988), sea urchins (Brandhorst, 1985) and insects (Raikhel, 1992; Zurita et al., 1997; Niu and Fallon, 1999, 2000). Using antisense-transformed flies, rpA1 and rp49 were shown to be critical for oogenesis in Drosophila melanogaster (Qian et al., 1988; Patel and Jacobs-Lorena, 1992), and a study using a D. melanogaster mutant demonstrated that rpS2 is essential for oogenesis (Cramton and Laski, 1994).
We recently reported a role for \textit{rpS3a} in regulating diapause, and hence ovarian development, in the mosquito \textit{Culex pipiens} (Kim et al., 2009). This gene is down-regulated for a few days at the onset of diapause, while it is expressed continuously in nondiapausing females. Suppression of \textit{rpS3} using RNAi mimicked a diapause-like arrest in nondiapausing females, and this suppression could be rescued with juvenile hormone, in much the same way that juvenile hormone can re-initiate ovarian development in diapausing females. The \textit{rpS3} results prompted these experiments designed to determine if other rps may also be selectively down-regulated during diapause in \textit{C. pipiens}. The well-documented role for \textit{rpS2} in regulating oogenesis in \textit{D. melanogaster} (Cramton and Laski, 1994), suggested merit in examining \textit{rpS2} as a potential contributor to the diapause response. \textit{C. pipiens} enters an adult reproductive diapause that is characterized by a much slower rate of ovarian development than observed in nondiapausing females (Christophers, 1911; Spielman and Wong, 1973b), thus in some manner, the progression of ovarian development observed in nondiapausing females is severely retarded by the diapause program. This diapause, induced by short daylengths and low temperatures (Sanburg and Larsen, 1973; Spielman and Wong, 1973a; 1973b), is initiated and maintained at the endocrine level by a shut-down in juvenile hormone (JH) production (Readio and Meola, 1985; Readio et al., 1999), as is also the case for most diapauses occurring in
the adult stage (Denlinger et al., 2005).

In this study, we observe the expression pattern of \textit{rpS2} in long-day (nondiapause) and short-day (diapause) females of \textit{C. pipiens} that were reared at the same temperature (18°C) and note a conspicuous diapause-specific drop in \textit{rpS2} expression 10-18 days after adult eclosion. We then attempt to mimic this effect in nondiapausng females using RNA interference and demonstrate that JHIII can rescue the suppression we observed following RNAi. The results we present are consistent with a role for \textit{rpS2} in the network of genes regulating diapause in \textit{C. pipiens}.

\textbf{Materials and Methods}

\textit{Insect rearing}

The colony of \textit{C. pipiens} (Buckeye strain) was maintained at 25°C, 75% R.H., with a 15L (light):9D (dark) cycle (Nondiapause, 25°C) as described in Robich and Denlinger (2005). As second instar larvae, experimental groups were transferred to either 18°C, 75% R.H., 9L:15D to program diapause (Diapause, 18°C) or to 18°C, 75% R.H., 15L:9D as a nondiapause control group (Nondiapause, 18°C). Adult mosquitoes were provided water and a 10% sucrose solution.
Cloning, RNA extraction, and cDNA preparation

Sequences of genes encoding rpS2, and rpL19 as a control/reference gene, were retrieved from the *C. quinquefasciatus* genome database on VectorBase (www.vectorbase.org, CPIJ014473-RA and CPIJ014540-RA) and compared to sequence identities of *Aedes aegypti* retrieved from the BLAST program of the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). Both genes were cloned using a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) with forward and reverse primers (rpS2: 5’-TTG ACG TAG CGG AGA CAC AC-3’ and 5’-AAG GTC GCT TGC ATT GTT TT-3’; rpL19: 5’- ATG AGT TCC CTC AAG CTC CA-3’ and 5’-ATC AGG ATG CGC TTG TTC TT-3’).

Total RNAs were extracted from female mosquitoes using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Absorbance was measured to quantify RNA by a spectrophotometer at 260nm (BioSpecmini, Shimadzu, Kyoto, Japan). For experiments that examined ovaries, RNA from 50 ovaries (25 females) was collected for each condition. cDNAs were prepared from 5µg of total RNA using Oligo(dT)20 Primers and SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.
**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

The prepared cDNAs were amplified using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with 10µM of forward and reverse primers of *rpS2* or *rpL19* and PCR SuperMix (Invitrogen, Carlsbad, CA, USA) using the following conditions: 94°C for 3 min, 25 cycles of 94°C for 30 sec/60°C for 30 sec/72°C for 3 min, and 72°C for 7 min. Electrophoresis using a 1% agarose gel was run to check the RT-PCR products.

**Quantitative real-time PCR (qPCR)**

qPCR was performed using an iQ™5 Multicolor Real-time PCR Detection system (BioRad, Hercules, CA, USA). Standard curves for *rpS2* and *rpL19* with 5µM of forward and reverse primers (*rpS2*: 5’-AAG ACG CCG TAC CAG GAG TA-3’ and 5’-AGG TCG CTT GCA TTG TTT TT-3’; *rpL19*: 5’-CGC TTT GTT TGA TCG TGT GT-3’ and 5’-CCA ATC CAG GAG TGC TTT TG-3’) were generated using 8 dilution series by plasmid copy number. Reactions with a total volume of 20µl (10µl of iQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA), 2µl of cDNA, 2µl of each 5µM primer, and dH2O) were run at the following conditions: 95°C for 3 min, 40 cycles of 95°C for 15 sec/60°C for 30 sec/72°C for 30 sec, and 95°C for 1 min. Each reaction was performed in triplicate using 2 biological
replicates. *RpL19* was used to normalize expression levels as a loading control.

*dsRNA synthesis and RNAi experiments*

dsRNAs of *rpS2, rpL19*, and *β-galactosidase* (a second control) were synthesized using the MEGAscript T7 transcription kit (Ambion, Applied Biosystems, Foster City, CA, USA) as previously described (Sim et al., 2007). T7-forward and reverse primers used to prepare dsRNAs were as follows: *rpS2* 5’-TAA TAC GAC TCA CTA TAG GGT TGA CGT AGC GGA GAC ACA C-3’ and 5’-TAA TAC GAC TCA CTA TAG GGA AGG TCG CTT GCA TTG TTT T-3’; *rpL19* 5’-TAA TAC GAC TCA CTA TAG GGA TGA GTT CCC TCA AGC TCC A-3’ and 5’-TAA TAC GAC TCA CTA TAG GGA TCA GGA TGC GCT TGT TCT T-3’; *β-gal* 5’-TAA TAC GAC TCA CTA TAG GGG TCG CCA GCG GCA CCG CGC CTT TC-3’ and 5’-TAA TAC GAC TCA CTA TAG GGG TCG CCA GCG GCA CCG CGC CTT TC-3’. 0.8 µg/µl dsrpS2 or 0.5 µg/µl dsrpL19 or dsβ-gal was injected into the thorax of cold, carbon dioxide-anesthetized nondiapauing female mosquitoes (reared at 18°C) 3 or 5 days after eclosion using a microinjector (Tritech Research, Los Angeles, CA, USA). After injection all females were held at 18°C, 75% R.H., 15L:9D and provided a 10% sucrose solution. Ovaries were dissected 2, 4, 7, and 11 days after injection, and the lengths of primary follicles were measured using an
Inverted Tissue Culture Microscope (Nikon Diaphot, Melville, New York, USA). To verify RNAi efficiency, RT-PCR was performed as described above. One-way ANOVA using MINITAB (Minitab Inc., version 14, State College, PA, USA) was used to analyze statistical significance of differences in lengths of the primary follicles. A pair-wise comparison was used for multiple treatment comparisons, and a 5% significance level was applied to all tests.

Neutral red staining and microscopy

Neutral red solution (0.5% w/v, acetate buffer, pH 5.2, N6264, Sigma-Aldrich, St. Louis, MO, USA) was used to stain the follicles. Ovaries were dissected and immediately rinsed in insect saline (Ringer, 1883). They were then placed in neutral red solution for 10 sec and rinsed by dropping insect saline onto the slide glass (Clements and Boocock, 1984). Images of follicles were prepared using a Zeiss Axioskop Widefield Light Microscope at the Campus Microscopy and Imaging Facility at The Ohio State University.

Juvenile hormone (JH) III application

JHIII (Sigma-Aldrich, St. Louis, MO, USA) was applied to determine whether it could rescue the arrested ovarian development that occurred in response to
a dsrpS2 injection. 0.1µg/µl JHIII diluted in acetone was topically applied to the nondiapausing females immediately after dsrpS2 injection. Lengths of primary follicles were measured 2, 4, and 7 days after dsrpS2 injection/JHIII application.

Results

Low expression of rpS2 in diapausing females

Expression of rpS2 in nondiapausing and diapausing females was first determined by RT-PCR (Figure 1 A). In nondiapausing mosquitoes, rpS2 remained continuously expressed at high levels from 1 day to 1 month after adult eclosion. By contrast, expression of rpS2 was conspicuously low in diapausing females on days 10-18. qPCR result confirmed low expression in early diapause, as shown in Figure 1 B. The transcriptional level of rpS2 was low in early diapause (day 5) compared to the level in nondiapausing females, was even lower by day 15, and then again increased by 1 month. Thus both indices of expression consistently showed low expression of rpS2 during the early phase of diapause. The above results are based on expression in whole bodies, but the same distinction was noted in ovaries dissected from mosquitoes on day 14 (Figure 2 A).
Suppression of oogenesis caused by dsrpS2 injection

dsrpS2 was injected into nondiapausing mosquitoes to evaluate the role of

rpS2 in oogenesis. When primary follicle lengths were measured 2, 4, 7, and 11 days
after dsrpS2 injection into 3 day-old nondiapausing females, follicles were

significantly smaller than those in control mosquitoes of the same age (Figure 3 A).
Arrested ovarian development caused by dsrpS2 was also shown in 5 day-old

nondiapausing mosquitoes, even though the follicles were larger because of their

initial growth prior to injection. As more time elapsed after injection, follicle length

slightly increased, possibly a consequence of dsRNA degradation, as noted in other

RNAi experiments (Byrom et al., 2002; Dorn et al., 2004; Hemmings-Mieszczak et

al., 2003; Kim et al., 2009). Two internal controls, dsrpL19 and dsβ-gal, were also

evaluated, and neither showed suppression in size of the follicles (Figure 3 A). A

pair-wise comparison showed significant differences between the nondiapause

control group and the dsrpS2-injected group, and between the dsrpS2-injected group

and the dsrpL19-injected group (P < 0.05). The dsRNA suppression of genes

encoding rpS2 and rpL19 was confirmed by RT-PCR (Figure 3 B). The suppression

of rpS2 within the ovaries was also tested, and qPCR results show that rpS2 was

suppressed within the ovaries 2 days after dsrpS2 was injected into day 3 and 5

nondiapausing mosquitoes (Figure 2 B). The physical appearance of the primary
follicles stained by neutral red staining solution for each condition is shown in Figure 4. These results are consistent in showing that suppression of \( rpS2 \) arrests oogenesis, thus suggesting that the naturally-occurring reduction in \( rpS2 \) associated with diapause may be an important event regulating the observed arrest in oogenesis that characterizes diapause.

\textit{JHIII rescues the arrest in oogenesis}

JHIII was applied to evaluate its ability to terminate the arrested oogenesis caused by dsrpS2 injection. In response to JHIII, follicles that were suppressed by dsrpS2 again initiated development (Figure 5). Two days after a dsrpS2 injection was coupled with JH application, follicle length was slightly greater than in dsrpS2-injected females that did not receive a JHIII application. Four and seven days after receiving an injection coupled with JHIII, follicle lengths were much greater in females injected at both 3 and 5 days. These results indicate that JHIII can rescue the arrested ovarian development caused by dsrpS2 injection.

Discussion

Our results show that expression of \( rpS2 \) is shut down for 2-3 weeks, beginning approximately 5-10 days after eclosion, in adults of \textit{C. pipiens} that have
been reared under the short daylengths that program the females for a reproductive diapause, while females reared under the long daylengths that promote immediate egg maturation express \textit{rpS2} continuously. The fact that RNAi directed against \textit{rpS2} in nondiapausing females suppresses development of the follicles in a manner akin to diapause suggests a functional link between the expression pattern of this gene and the developmental arrest observed during diapause. In addition, the observation that JHIII, the form of JH used by mosquitoes, can rescue the arrest elicited by dsrpS2, just as it can terminate diapause in this species, further strengthens the argument that a shut-down in \textit{rpS2} is one of perhaps many genes that is involved in evoking the diapause response. Many genes contribute to the diapause response of \textit{C. pipiens} (Kim et al., 2006; Kim et al., 2009; Robich and Denlinger, 2005; Robich et al., 2007; Sim and Denlinger, 2008), thus we were not surprised that knocking down \textit{rpS2} does not arrest development for many months as is typical of diapause. In addition, the dsRNA is expected to degrade with time (Byrom et al., 2002; Dorn et al., 2004; Hemmings-Mieszczak et al., 2003; Kim et al., 2009) and thus can not be expected to continuously suppress the gene of interest.

One other gene encoding a ribosomal protein in \textit{C. pipiens}, \textit{rpS3a}, displays a similar, yet distinct, expression pattern and response to RNAi (Kim et al., 2009; Robich et al., 2007). Expression of both \textit{rpS2} and \textit{rpS3a} is shut down at
approximately the same time, day 5-10 after eclosion, in diapausing females, but expression of \textit{rpS3a} again rebounds after being shut down for a few days, while the shut down in expression of \textit{rpS2} is much longer, persisting until the female is nearly one month old. Such a difference in the temporal profile of expression suggests distinct roles for these two \textit{rps} in bringing about the diapause response. The fact that suppressing either \textit{rpS3a} or \textit{rpS2} with RNAi can halt development, at least for a short time, suggests that they are both major players in the diapause response.

Presumably the arrest would be even more dramatic if both genes were suppressed at the same time, as is the case in natural diapause. A number of genes in other insects are in this same category of being either uniquely expressed or specifically not expressed in early diapause (Denlinger, 2002). We suspect that such genes may be especially important at the onset of diapause for bringing development to a halt.

In \textit{D. melanogaster}, the gene encoding \textit{rpS2} is referred to as \textit{string of pearls} (SOP) and is required for successful oogenesis (Cramton and Laski, 1994). In \textit{sop}\textsuperscript{p} mutants, oogenesis proceeds normally until the oocytes reach stage V and then they develop no further. We propose that the developmental arrest we see in diapause is a similar phenotype and may have a similar basis with a functional link to \textit{rpS2}.

Clearly, this is not the only gene contributing to diapause, but it is likely to be one of many downstream genes in the network of genes that program the diapause response.
Insulin signaling is thought to be a key regulator of diapause in *C. pipiens*, presumably upstream of JH production (Sim and Denlinger, 2008). The fact that JH can reverse the halt in development caused by dsRNA directed against *rpS2* suggests that the follicle cells are either directly or indirectly responsive to JH and that the high production of JH that would have been expected in nondiapausing females (Readio et al., 1999) was for some reason reduced when *rpS2* was suppressed.

Obviously we are seeing only pieces of the puzzle at this point, but we anticipate that any future comprehensive models for the regulation of reproductive diapause in *C. pipiens* will need to be reconciled with the expression patterns and responses we have observed for *rpS2* and *rpS3a*. 
References


Kim, M., Sim, C. and Denlinger, D.L. (2009) RNA interference directed against ribosomal protein S3a suggests a link between this gene and arrested ovarian development during adult diapause in Culex pipiens. Insect Molecular Biology (In press).


Ringer, S. (1883) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. The Journal of Physiology 4:29-42.


Figure 4.1. Expression of \textit{rpS2} (\textit{ribosomal protein S2}) in nondiapausing and diapausing females of \textit{Culex pipiens}. (A) RT-PCR (Reverse Transcription-Polymerase Chain Reaction) demonstrates that \textit{rpS2} is continuously expressed from 1 day to 1 month after adult eclosion in nondiapausing females, but is down-regulated on days 10-22 in diapausing females. \textit{RpL19} (\textit{ribosomal protein L19}) was used as a control. (B) qPCR (Quantitative real-time PCR) confirmed the above transcriptional levels of \textit{rpS2}. Compared to nondiapausing females (ND), the transcriptional level of \textit{rpS2} was lower in diapausing females in early diapause (day 5), lowest in diapausing females on day 15, and high again in diapausing mosquitoes.
at 1 month of age. *RpL19* was used to normalize expression levels as a loading control. Error bars represent standard deviations (N = triplicate of two biological samples).
Figure 4.2. Ovarian expression of \( rpS2 \), based on qPCR. (A) \( RpS2 \) expressed highly in ovaries of nondiapausing mosquitoes (ND) 14 days after adult eclosion, but low in those of diapausing mosquitoes (D) on day 14. (B) Suppression of \( rpS2 \) in the ovaries in response to RNAi. Injection of dsrpS2 was made into 3 and 5 day-old females and ovarian expression was measured 2 days later (days 5 and 7). \( RpL19 \) was used to normalize expression levels as a loading control. Error bars represent standard deviations (N = triplicate of two biological samples). d = days after adult eclosion, dai = days after dsRNA injection.
Figure 4.3. The effect of dsrpS2 injection on follicle length. (A) Mean±SD length of primary follicles was measured (10 follicles measured per mosquito; total 10 mosquitoes for controls and 20 mosquitoes for others). The injected dsrpS2 suppressed ovarian development in nondiapausing mosquitoes. dsrpL19 and dsβ-gal were injected as internal controls. ND = Nondiapausing mosquitoes, D = Diapausing mosquitoes, d = days after adult eclosion, dai = days after dsRNA injection. Numbers after ND and D refer to age of females, in days, at the time of injection. All ND dsrpS2-injected groups were significantly different from ND, and dsrpL19 and
dsβ-gal control groups measured at the same age, but were not significantly different from the D group (P > 0.05). (B) Suppression of *rpS2* and *rpL19* in nondiapauing mosquitoes after injection of dsrpS2 and dsrpL19, confirmed by RT-PCR.
Figure 4.4. Primary follicles from nondiapausing (Top) and diapausing (Middle) females stained with neutral red on days 5 and 7 after eclosion. Bottom panels show primary follicles 2 days after dsrpS2 injection into day 3 and 5 nondiapausing.
females. (Scale bar = 50\(\mu\)m)
Figure 4.5. Juvenile hormone III rescues the suppression of oogenesis caused by injection of dsrpS2 into nondiapausing females. Mean±SD primary follicle length was measured (10 follicles measured per mosquito; total 10 mosquitoes for controls and 20 mosquitoes for others). Oogenesis was initiated when JHIII was applied immediately after dsrpS2 injection. ND = Nondiapausing mosquitoes, D = Diapausing mosquitoes, d = days after adult eclosion, dai = days after dsrpS2 injection. Numbers after ND and D refer to age of females, in days, at the time of injection. All ND dsrpS2-injected groups were significantly different from ND and JHIII treatment groups measured at the same age, but were not significantly different from the D group (P > 0.05).
Chapter 5: Interaction between heat shock protein 90 and ribosomal protein S6 in the northern house mosquito, Culex pipiens

Abstract

Heat shock protein (hsp90) was continuously expressed in nondiapausing females of Culex pipiens for at least 1 month after adult eclosion, but in females programmed by short day length for an overwintering diapause expression of hsp90 consistently declined for a short time around day 14. Hsp90 has previously been implicated in interactions with several ribosomal proteins (rp), and in this study we used RNA interference (RNAi) directed against hsp90 to evaluate the possibility that it alters expression of rpS6. Like hsp90, rpS6 was expressed continuously in nondiapausing females, but expression declined around day 14 in diapausing females. Thus, expression of both genes synchronously dipped 2 weeks after emergence in diapausing, but not in nondiapausing females. When dshsp90 was injected into nondiapausing mosquitoes, not only was hsp90 suppressed, but rpS6 was also suppressed. These results suggest an interaction between these two genes and the possibility that hsp90 controls expression of rpS6. The decline in expression of
hsp90 during diapause likely contributes to the shut-down of rps6 and possibly other ribosomal genes during the mosquito’s overwintering diapause.

Introduction

Though heat shock proteins are best known for their responses to stress they are a diverse group, and many are expressed constitutively and contribute to diverse cellular functions (Hendrick and Hartl, 1993). Heat shock protein 90 (hsp90) is among the hsps present in cells at all times, where it functions as a chaperone regulating activity of specific signaling proteins and transcription factors (Vabulas et al., 2002) and interacts with ribosomal proteins to facilitate their formation and to optimize their function (Fromont-Racine et al., 2003; Leary and Huang, 2001). Several reports document the expression of hsp90 in relation to insect diapause, especially larval and pupal diapauses, but the responses differ from species to species. In Sarcophaga crassipalpis, hsp90 expression declines 2 days after pupariation (before the entry into pupal diapause) (Hayward et al., 2005), remains low throughout-diapause and then increases again at diapause termination (Rinehart and Denlinger, 2000). This is in contrast to other hsps that are developmentally up-regulated during diapause (Rinehart et al., 2007). As in the pupal diapause of S. crassipalpis, expression of hsp90 increases at diapause termination in larvae of the
blow fly, *Lucilia sericata* (Tachibana et al., 2005). By contrast, hsp90 is up-regulated during larval diapause in *Chilo suppressalis* (Sonoda et al., 2006).

Hsp90 is known to interact with several ribosomal proteins (rps), including rpS6. RpS6 is part of a small ribosomal subunit that functions in translation and controls cell growth and proliferation (Meyuhas, 2008). In the mammalian target of rapamycin (mTOR)-raptor signaling pathway, hsp90 affects protein translation by the phosphorylation of both eukaryotic initiation factor 4E (eIF4E) binding protein 1 and ribosomal p70 S6 kinase (Ohji et al., 2006). Also hsp90 appears to regulate the stability of rpS3 and rpS6 as shown by hsp90-rpS3 and hsp90-rpS6 interactions that prevent ubiquitination and the proteasome-dependent degradation of rpS3 and rpS6 (Kim et al., 2006).

Only a few studies report expression patterns for *rpS6* among invertebrates. In *Aedes aegypti*, mRNA of rpS6 is up-regulated 1-2 days after adult eclosion and remains high until the female mosquitoes take a blood meal and initiate egg production (Niu and Fallon, 2000). In the brine shrimp, *Artemia franciscana*, *rpS6 kinase*, the gene encoding an enzyme required for rpS6 activation, increases rapidly when embryos break diapause and are ready to hatch (Santiago and Sturgill, 2001). In a previous report from our laboratory, Robich et al. (2007) reported that the gene encoding rpS6 in *Culex pipiens* is expressed at a low level in early diapause but is
highly expressed late in diapause.

In this study, we examine expression of hsp90 and rpS6 in both nondiapausing and diapausing female mosquitoes of C. pipiens, and the relationship between these two genes is examined by monitoring the expression of rpS6 following the use of RNAi to knock down the expression of hsp90. We report that both genes have similar expression patterns, reflected in a synchronous drop in expression during early diapause and that suppression of hsp90 elicits a simultaneous decline in the expression of rpS6.

Materials and Methods

Insect rearing

The colony of C. pipiens (Buckeye strain) was maintained at 25°C, 75% R.H., with a 15L (light):9D (dark) cycle (Nondiapause, 25°C) as described in Robich and Denlinger (2005). For our experiments, second instar larvae were transferred to either 18°C, 75% R.H., 9L:15D to program diapause (Diapause, 18°C) or to 18°C, 75% R.H., 15L:9D as a nondiapause control group (Nondiapause, 18°C). Adult mosquitoes were provided water and cotton soaked in 10% sucrose solution.
Cloning, RNA extraction, and cDNA synthesis

Primers for rpS6 were designed from the retrieved sequences reported by Robich et al. (2007) and the A. aegypti rpS6 sequence from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) using BLAST programs. Sequences of genes encoding hsp90 and rpL19 (a control gene) were from the C. quinquefasciatus genome database on VectorBase (www.vectorbase.org) and compared to sequence identities of A. aegypti retrieved from the BLAST program.

All genes were cloned using a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) with forward and reverse primers (rpS6: 5’-AGA AGG GCC ATT CGT GCT AC-3’ and 5’-TCC TTC TTG GCC TCC TTC TT-3’; hsp90: 5’-GAA GCA CTT CTC CGT TGA GG-3’ and 5’-AGT TCC TCG AAC AGC TCC AA-3’; rpL19: 5’-ATG AGT TCC CTC AAG CTC CA-3’ and 5’-ATC AGG ATG CGC TTG TTC TT-3’). Total RNAs were extracted from female mosquitoes using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.

Absorbance was measured to quantify RNA by a spectrophotometer at 260nm (BioSpecmini, Shimadzu, Kyoto, Japan). cDNAs were prepared from 5µg of total RNA using Oligo(dT)$_{20}$ Primers and SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.
Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The prepared cDNAs were amplified using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with 10µM of forward and reverse primers for \textit{rpS6}, \textit{hsp90} or \textit{rpL19} and PCR SuperMix (Invitrogen, Carlsbad, CA, USA) using the following conditions: 94°C for 3 min, 25 cycles of 94°C for 30 sec/60°C for 30 sec/72°C for 3 min, and 72°C for 7 min. Electrophoresis using a 1% agarose gel was run to check the RT-PCR products. RT-PCR was repeated twice using biological replications.

Northern blot hybridization

Formaldehyde denaturing RNA gels (4µg of RNA was used for each condition) were transferred onto a Hybond-nylon positive membrane (GE Healthcare Bio-Science, Uppsala, Sweden) using a Schleicher and Schuell TurboBlotter system (Schleicher and Schuell BioScience Inc, Keene, NH, USA). The \textit{hsp90} probe was labeled using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences, Indianapolis, IN, USA). 28S ribosomal RNA from \textit{C. pipiens} was used to verify equal loading. All Northern blot hybridization results were replicated three times with independent samples.
**RNAi experiments**

dshsp90 was synthesized using the MEGAscript T7 transcription kit (Ambion, Applied Biosystems, Foster City, CA, USA) as previously described (Sim et al., 2007). T7-forward and reverse primers used to prepare dshsp90 were 5’-TAA
TAC GAC TCA CTA TAG GGG AAG CAC TTC TCC GTT GAG G-3’ and 5’-TAA
TAC GAC TCA CTA TAG GGA GTT CCT CGA ACA GCT CCA A-3’. 1µg/µl
dshsp90 was injected into the thorax of cold, carbon dioxide-anesthetized
nondiapausing female mosquitoes (reared at 18°C) 7 or 10 days after eclosion using
a microinjector (Tritech Research, Los Angeles, CA, USA). After injection all
females were held at 18°C, 75% R.H., 15L:9D and provided a 10% sucrose solution.
To verify RNAi efficiency, RT-PCR was performed as described above.

**Results**

*Low mRNA level of rpS6 and hsp90 in diapausing female mosquitoes*

mRNA levels of *rpS6* and *hsp90* in nondiapausing and diapausing
mosquitoes reared at 18°C were quantified by RT-PCR (Figure 1 A). *RpS6* and *hsp90*
were consistently expressed from 1 day to 1 month after adult eclosion in
nondiapausing mosquitoes, but in diapausing mosquitoes all replicates indicated a
simultaneous down-regulation of *rpS6* and *hsp90* on day 14. Figure 1 B shows a
finer time scale for the expression of \textit{rpS6} and \textit{hsp90} in diapausing mosquitoes.

Again, both \textit{rpS6} and \textit{hsp90} were less highly expressed on day 14. Low expression of \textit{rpS6} was still evident on day 18, but expression of \textit{hsp90} rebounded by that time.

Expression of the control gene, \textit{rpL19} remained similar for nondiapausing and diapausing females of all ages. The lower expression of \textit{hsp90} at 14 days after adult eclosion in diapausing females was also confirmed by Northern blot hybridization (Figure 1 C): \textit{hsp90} expression was high in nondiapausing females reared at 25°C (ND25) and 18°C (ND18) but was much lower in diapausing females of the same age, reared at 18°C (D18). Thus, both \textit{rpS6} and \textit{hsp90} were down-regulated in diapausing females at the same time, although the down-regulation of \textit{rpS6} persisted longer than the down-regulation of \textit{hsp90}. An earlier report on \textit{rpS6} expression in \textit{C. pipiens} (Robich et al., 2007) indicated low levels of expression in both nondiapausing females and in females collected in early diapause, and high expression late in diapause. The previous work was based on a short (296bp) clone derived from SSH, while we used a much longer (1777bp) clone in this study. We attribute these differences to the higher sensitivity of this larger clone and the more comprehensive developmental time frame used in the current experiments.
**dshsp90 suppressed rpS6 expression**

dshsp90 was injected into nondiapausing mosquitoes to evaluate its impact on rpS6 expression. When dshsp90 was injected into 7 and 10 day-old females, expression of both hsp90 and rpS6 was suppressed, whereas the control gene, rpL19 was consistently expressed (Figure 2). This result suggests that hsp90 may influence the transcription of rpS6.

**Discussion**

Our results suggest a potential interaction between hsp90 and rpS6, an interaction consistent with results noted in human kidney cells, where hsp90 inhibitors negatively impacted levels of rpS6 (Kim et al., 2006). The expression patterns of hsp90 and rpS6 were similar. Both genes were continuously present in nondiapausing females of C. pipiens, but in diapausing females expression of both genes simultaneously declined on day 14. When RNAi was directed against hsp90 in nondiapausing females, the knock-down of hsp90 was accompanied by a similar suppression of rpS6, thus suggesting a link between these two genes and the possibility that hsp90 affects the transcription of rpS6. Numerous genes are suppressed during diapause or at least for select phases of diapause (Denlinger, 2002; Kim et al., 2009), and these results with hsp90 suggest one of the interactions that
may lead to suppression of multiple genes during diapause.

These results also underscore the multiple roles played by hsps during diapause. The up-regulation of \textit{hsp70} and small \textit{hsps} during diapause is reported for quite a few species, and at least in \textit{S. crassipalpis}, the abundances of these \textit{hsps} contribute to overwintering cold tolerance (Rinehart et al., 2007). But, as evidenced in \textit{S. crassipalpis}, the role for \textit{hsp90} during diapause is quite different; rather than being up-regulated, \textit{hsp90} is down-regulated throughout diapause (Rinehart and Denlinger, 2000). And, in other species, \textit{hsp90} can be either up (Chen et al., 2005) or down (Goto and Kimura, 2004; Tachibana et al., 2005) during diapause. The pattern we now see in \textit{C. pipiens} represents yet another unique expression pattern for \textit{hsp90} during insect diapause. Rather than being consistently up- or down-regulated during diapause, \textit{hsp90} is down-regulated only briefly during diapause in this species. Though this down-regulation could easily be overlooked due to its brevity, the down-regulation of \textit{hsp90} around 14 days was consistently observed in all replicates, and the RNAi results suggest a link to the suppression of \textit{rpS6}.
References


Kim, M., Sim, C. and Denlinger, D.L. (2009) RNA interference (RNAi) of ribosomal protein S3a (rpS3a) suggests a link between this gene and arrested ovarian development during adult diapause in \textit{Culex pipiens}. Insect Molecular Biology (In press)


Figure 5.1. Quantification of mRNAs encoding rpS6 (ribosomal protein S6) and hsp90 (heat shock protein 90) in diapausing and nondiapausing females of *Culex pipiens*. (A) RT-PCR was used to detect and quantify mRNA levels of rpS6, hsp90, and a control gene, *rpL19* (ribosomal protein L19) in nondiapausing (long daylength) and diapausing (short daylength) mosquitoes reared at 18°C. Both *rpS6* and *hsp90* were continuously expressed in nondiapausing mosquitoes after adult eclosion, but *rpS6* and *hsp90* were expressed at lower levels on day 14 in diapausing mosquitoes. (B) RT-PCR showing further evidence for a brief suppression of *rpS6* and *hsp90*
expression on day 14 in diapausing females. Low expression persisted for *rpS6* through day 18, but rebounded by day 22. Low expression of *hsp90* was evident only on day 14. (C) Northern blot hybridization confirmed the down-regulation of *hsp90* at 14 day in diapausing females. ND25: nondiapausing females reared at 25°C, ND18: nondiapausing females reared in 18°C, D18: diapausing females reared at 18°C. When temperatures are not indicated, all mosquitoes used in experiments were reared at 18°C.
Figure 5.2. The effect of dshsp90 injection into nondiapausing mosquitoes reared at 18°C. The dshsp90 was injected into 7 and 10 day-old female mosquitoes and expression was evaluated 3 or 4 days after injection (dai). RNA interference (RNAi) directed against hsp90 suppressed expression of both hsp90 and rpS6. The control gene, rpL19, was not affected by the dshsp90 injection.
Conclusions

This study examined the expression patterns of several gene groups that show unique patterns during diapause in *Culex pipiens*. Cytoskeletal genes and genes encoding ribosomal proteins were cloned for expression in nondiapausing and diapausing female mosquitoes using Northern blot hybridization, RT-PCR, or qPCR, and RNAi was performed on two of the ribosomal protein genes to investigate functions. These genes are among those involved in controlling diapause, and the results are linked with reproductive function and low temperature response.

I. Decrease in expression of *beta-tubulin* and microtubule abundance in flight muscles during diapause in adults of *Culex pipiens*

   1. The full-length cDNA of *beta-tubulin* is 1395bp, encoding 443 amino acids. *Beta-tubulin* has high similarity with the *beta-tubulin* genes in other insects.

   2. Among three different body regions (head, thorax, abdomen), *beta-tubulin* showed much less expression in the thorax during early and mid diapause.
Since the thorax is mostly composed of flight muscles, a down-regulation of 

*beta-tubulin* in diapausing mosquitoes may relate to lower flight activity 
during diapause.

3. The abundance and structural patterns of microtubules in midguts and 
thoracic muscles of nondiapausing and diapausing females were evaluated. 
In midguts, the microtubule abundance and structural patterns were similar 
in nondiapausing and diapausing females, whereas in thoracic muscles 
microtubule abundance was significantly less in diapausing females, even 
though the patterns were similar. This result supports the hypothesis that low 
expression of *beta-tubulin* in flight muscles is linked to diapause in *C. pipiens*.

4. When microtubule abundance in midguts was examined after low 
temperature exposure, the tubulin level dramatically decreased after 
exposure to 0°C and was even lower after exposure to -5°C in nondiapausing 
females. However, it was already low in midguts of diapausing females and 
remained low after exposure to 0°C and -5°C. Similar patterns in 
nondiapausing and diapausing females were evident in thoracic muscles. 
Low temperature exposure affects changes in tubulin abundance, but also the 
diapause program influences lower abundance in the thoracic muscles.
II. RNA interference directed against ribosomal protein S3a suggests a link between this gene and arrested ovarian development during adult diapause in Culex pipiens

1. *RpS3a* was continuously expressed at least until 2 months after adult eclosion in nondiapausing female mosquitoes, but it was down-regulated in 7-10 day old diapausing females. These patterns were evident in both whole body samples as well as in the ovaries.

2. RNAi directed against rpS3a was performed to evaluate the function of this gene and ovarian development. When dsrpS3a was injected into nondiapasing females, the primary follicles were arrested in their development, a response that mimics diapause. In contrast, follicles grew normally after injection of the controls, dsrpL19 and dsβ-gal.

3. The RNAi effect persisted approximately 10 days after dsRNA injection. Degradation of the injected dsRNA limits the duration of the RNAi effect.

4. When JHIII, the endocrine trigger that terminates diapause in this species, was applied immediately after a dsrpS3a injection into nondiapausng females, the arrested follicles initiated development after 2-4 days. The fact that JHIII can rescue the arrested ovarian development caused by dsrpS3a further suggests a link between the function of rpS3a and diapause.
III. A potential role for ribosomal protein S2 in the gene network regulating reproductive diapause in the mosquito *Culex pipiens*

1. The gene encoding rpS2 showed continuous expression from 1 day to 1 month after adult eclosion in nondiapausing female mosquitoes, whereas this gene showed a drop in expression on day 5-18 in diapausing females. Results were consistent in both whole body and ovarian samples.

2. dsrpS2 was injected into nondiapausing females to evaluate its potential effect on reproduction. Suppression of *rpS2* arrested ovarian development, thus mimicking diapause. As in the *rpS3a* experiment, dsrpL19 or dsβ-gal did not affect follicle development. These results suggest that *rpS2* has a role in suppressing ovarian development during diapause in *C. pipiens*.

3. A topical application of JHIII after a dsrpS2 injection into nondiapausing females was able to rescue the arrested ovarian development elicited by a dsrpS2 injection, thus suggesting a link between *rpS2* and juvenile hormone in the regulation of diapause.

IV. Interaction between heat shock protein 90 and ribosomal protein S6 in the northern house mosquito, *Culex pipiens*
1. Expression of \textit{hsp90} and \textit{rpS6} was examined using RT-PCR and Northern blot hybridization. Both genes were expressed continuously from 1 day to 1 month in nondiapausing female mosquitoes, but both genes were simultaneously down-regulated on day 14 in diapausing mosquitoes, \textit{rpS6} showed further down-regulation on day 18.

2. When \textit{dshsp90} was injected into nondiapausing female mosquitoes to investigate a relationship with \textit{rpS6}, both \textit{hsp90} and \textit{rpS6} were suppressed, but \textit{rpL19}, the control gene was not affected. This RNAi experiment suggests that \textit{hsp90} has a possible role in controlling the transcription of \textit{rpS6}.
Appendix A: Low temperature elicits expression of ribosomal protein S13 (rpS13) in nondiapausing adults of the northern house mosquito, Culex pipiens

Introduction

Ribosomal protein S13 (rpS13) is one of the proteins located in the small ribosomal subunit. RpS13 is known to be involved in the initiation of translation by binding fMET-tRNA in Escherichia coli (Chan et al., 1991). Also, in human rpS13, it regulates its own expression during splicing by a feedback mechanism (Malygin et al., 2007).

Several types of cold-sensitive mutants in yeast show a deficiency in the assembly in ribosomal subunits (Bayliss and Ingraham, 1974; Hartwell et al., 1970), implying that, as part of the low temperature response, protein synthesis and the translation machinery are required. In the soybean, Glycine max, genes encoding three ribosomal proteins, rpS13, rpS6, and rpL37 are induced by low temperature (Kim et al., 2004).

In this study, we show that rpS13 is induced by low temperature in nondiapausing females of Culex pipiens. Also, we show that the gene is expressed...
differently after low temperature treatment in diapausing females.

Materials and Methods

Insect rearing

The colony of C. pipiens (Buckeye strain) was maintained at 25°C, 75% R.H., with a 15L (light):9D (dark) cycle (Nondiapause, 25°C) as described in Robich and Denlinger (2005). As second instar larvae, experimental groups were transferred to either 18°C, 75% R.H., 9L:15D to program diapause (Diapause, 18°C) or to 18°C, 75% R.H., 15L:9D as a nondiapause control group (Nondiapause, 18°C). Adult mosquitoes were provided water and a 10% sucrose solution. Only females were used in this study.

Low temperature exposure

7 and 10 day-old nondiapausing and diapausing mosquitoes within 50ml centrifuge tubes (Fisher Healthcare, Houston, TX) were exposed to low temperatures in a water bath, Phoenix II (C35P) (Thermo Scientific, Waltham, MA) for 3-12 hours or in the cold room (4°C) for 1-7 days. 15 females were used in each treatment and each treatment was repeated twice. All mosquitoes were collected immediately after the low temperature exposure for RNA extraction.
RNA extraction and cDNA synthesis

Total RNA was extracted from female mosquitoes using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Absorbance was measured to quantify RNA by a spectrophotometer at 260nm (BioSpecmini, Shimadzu, Kyoto, Japan). cDNAs were prepared from 5 µg total RNA using Oligo(dT)\(_{20}\) Primers and SuperScript\(^{TM}\) III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions.

Quantitative real-time PCR (qPCR)

Sequences of genes encoding rpS13, and rpL19 as a reference gene, were retrieved from the *C. quinquefasciatus* genome database on VectorBase (www.vectorbase.org, CPIJ015099-RA and CPIJ014540-RA) and compared to sequence identities of *Aedes aegypti* retrieved from the BLAST program of the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). qPCR was performed using an iQ\(^{TM}\)5 Multicolor Real-time PCR Detection system (BioRad, Hercules, CA, USA). Standard curves for *rpS13* and *rpL19* with 5 µM of forward and reverse primers (*rpS13*: 5’-TAA GGA GAG ACG GGG AGG TT-3’ and 5’-TTT TCT CTC CCA CAT CG-3’; *rpL19*: 5’-CGC TTT GTT TGA TCG TTG TGT GT-3’ and 5’-CCA ATC CAG GAG TGC TTT TG-3’) were generated using 8
dilution series by plasmid copy number. Reactions with a total volume of 20µl (10µl of iQ™ SYBR® Green Supermix, BioRad, Hercules, CA, USA, 2µl of cDNA, 2µl of each 5µM primer, and dH2O) were run using the following conditions: 95°C for 3 min, 40 cycles of 95°C for 15 sec/60°C for 30 sec/72°C for 30 sec, and 95°C for 1 min. Each reaction was performed in triplicate using 2 biological replicates. *RpL19* was used as a loading control to normalize expression levels.

**Results and Discussion**

*RpS13 induced by low temperature in nondiapausing mosquitoes*

When nondiapausing female mosquitoes, day 7 after adult eclosion, were exposed to -5°C for 12 hours, the mRNA level of *rpS13* was elevated, while *rpS6* was reduced (Figure 1 A). Figure 2 depicts the response of *rpS13* to a range of low temperatures. On day 7, *rpS13* increased as the severity of low temperature increased (Figure 2 A). A similar pattern was noted on day 10, but the maximum increase was higher and was attained at less severe low temperatures (Figure 2 B). When 10 day-old nondiapausing females were placed at 4°C for various durations, *rpS13* gradually increased with duration of exposure (Figure 3 A).
**Downregulation of rpS13 in response to low temperature during diapause**

The response of *rpS13* to low temperature in diapausing mosquitoes was quite different. In diapausing females on day 7, *rpS13* expression level was much higher than in nondiapausing females of the same age (Figure 1 B). After exposure to -5°C for 12 hours, the mRNA level of *rpS13* in diapausing mosquitoes decreased, unlike the response noted in nondiapausing mosquitoes. *RpS6* showed a slightly higher expression after low temperature treatment in diapausing mosquitoes. When diapausing females were exposed to various durations of exposure to 4°C, the decline in expression was evident after 1 day of exposure and persisted throughout the 7 days of the experiment (Figure 3 B). Thus, rather than showing an increase in expression at low temperature, as seen in nondiapausing mosquitoes, the expression of *rpS13* declined in response to low temperature in diapausing mosquitoes.

In this study, we show that one of the ribosomal proteins, *rpS13*, is induced in response of low temperature. But, curiously this response was evident only in nondiapausing females, not in females that had entered diapause. The expression level of *rpS13* was already higher in diapausing females held at 18°C than in nondiapausing females held at the same temperature. This suggests that this gene is already up-regulated as a component of the diapause program. As documented for a
number of other cold-hardiness genes (Denlinger and Lee, 1998), a link to cold hardening is suggested by the up-regulation of this gene by cold in nondiapausing females.

However, it is not yet clear why expression would decline in response to cold in diapausing females, and the contribution of rpS13 to the low temperature response remains unclear. Perhaps it functions to enhance protein synthesis at low temperature and this may be more critical for nondiapausing than diapausing individuals (Kim et al., 2004). Further work on the relationship between rpS13 and the diapause program, the changes of rpS13 expression after low temperature exposure with recovery, and the translational level changes of rpS13 are required to better understand the function of rpS13.
References


Figure A.1. qPCR (Quantitative real-time PCR) results showing *rpS13* and *rpS6* transcriptional levels on day 7 after adult eclosion and after low temperature treatment at -5°C for 12 hours. (A) In nondiapausing mosquitoes, *rpS13* showed a higher transcriptional level after low temperature treatment. (B) In diapausing mosquitoes, *rpS13* was higher compared to nondiapausing controls, and the transcriptional level after low temperature treatment was much smaller. *RpS6* changed very little. *RpL19* was used as a loading control. Error bars represent standard deviations (N = triplicate of two biological samples).
Figure A.2. qPCR results of *rpS13* expression patterns in nondiapauasing females on (A) day 7 and (B) day 10 after adult eclosion and low temperature exposures of 0°C or -5°C for 3, 9, or 12 hours. The transcriptional level of *rpS13* on day 7 increased with time at 0°C, and was even higher in response to -5°C. On day 10, the patterns were similar to those on day 7, but *rpS13* was more responsive to low temperature.
Figure A.3. qPCR results showing rpS13 transcriptional levels in (A) nondiapausing and (B) diapausing females 10 days after eclosion, following exposure at 4°C for 1-7 days. (A) RpS13 expression in nondiapausing females gradually increased as a function of exposure time at 4°C. (B) Expression of rpS13 in diapausing females decreased in response to low temperature treatment and remained low for at least 7 days.
Bibliography


Farajollahi, A., Crans, W.J., Bryant, P.B., Wolf, B., Burkhalter, K.L., Godsey, M.S.,


Kim, M., Sim, C. and Denlinger, D.L. (2009) RNA interference (RNAi) of ribosomal protein S3a (rpS3a) suggests a link between this gene and arrested ovarian development during adult diapause in *Culex pipiens*. Insect Molecular Biology (In press)


Ringer, S. (1883) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. The Journal of Physiology 4: 29-42.


