ARMET TRANSCRIPT KNOCKDOWN IN *TRIBOLIUM CASTANEUM*

by

JARROD BRETT BECHARD

B.S. Kansas State University, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Biochemistry and Molecular Biophysics Graduate Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Approved by:

Major Professor
Gerald Reeck

Copyright

Abstract

Armet has been found in mammalian systems to be a bi-functional protein that is secreted extracellularly and is also found in the endoplasmic reticulum. It has been shown to be a neurotrophic factor and also a member of the unfolded protein response. Transcript knockdown of Armet via RNA interference in late instar larvae of *Tribolium castaneum* produces a fatal phenotype during eclosion from pupa to adult. Initial observations of pupae cuticle indicate disorganization of cuticles in insects with the Armet transcript knocked down. Here I expand studies on the effects of dsArmet RNA injection; both in a wild type strain and a fluorescent strain of Tribolium, and discuss possible mechanisms for the fatal phenotype.

Table of Contents

List of Figures

Acknowledgements

I would like to personally thank my academic advisor, Dr. Gerald Reeck, for his assistance and guidance throughout my graduate studies. I would also like to thank Dr. Raman Chandrasekar for all of his help with my research. I would like to thank Dr. John Tomich and Dr. David Wetzel for serving on my committee. I would like to thank Dr. Subbaratnam Muthukrishnan and Robbie Bear for access to resources in their respective labs. I would like to thank my lab colleagues, Matthew Aksamit and James Balthazor, for their support and assistance of my research. I would finally like to thank my family for their support as I have progressed through all of my studies here at Kansas State.

Chapter 1 - Armet Transcript Knockdown in *Tribolium castaneum*

Introduction

Tribolium castaneum

The red flour beetle, *Tribolium castaneum*, is a common pest in the grain industry. Making its home in stored grains such as flour and cereals, *T. castaneum* causes damage by feeding on grain and contaminating the overall stock. Contaminants include dead bodies, skin molts, liquids (quinones), and fecal pellets (Calvin, 1990). As a result, Tribolium-infested grains have a distinct, unpleasant odor and taste that deters livestock and potential buyers. Another consequence of Tribolium infestation involves their living conditions. The presence of these beetles in grain stock indicates that there may also be moisture and mold present as well, thus further reducing the quality and value of the product (Calvin, 1990).

The life cycle of *T. castaneum* takes 7 to 12 weeks for development from first instar larva to adult (Bennett, 2003). After hatching from the egg, there are four distinct stages in this life cycle: larva, pre-pupa, pupa, and adult (Figure 1.1). The larval stage can last between 22 and 100 days and the pupal stage approximately one week (Bennett, 2003). It is common for adults to live 1-3 years (Bennett, 2003).

Figure 1.1 Developmental stages of *T. castaneum*

The normal development of *T. castaneum* proceeds through four distinct stages. From left to right; final instar larva, pre-pupa, early pupa (< 2 days), late pupa (>4 days), young adult (< 1 week), and late adult (>1 week). Top panel shows larvae and adults with ventral side up. Bottom panel shows final instar larva and adults presented with dorsal side up. Pre-pupa is shown on its side to illustrate banana shape.

Cuticle Organization in Insects

The cuticle of insects has a wide range of functions which contribute to the overall success and survival of insects. These functions include giving structure to the insect, protecting the insect from mechanical and chemical damage, preventing water loss, and serving as a site for muscle and wing attachment (Keeley, 2004). The insect cuticle can be divided into two segments; the epicuticle and the procuticle. The epicuticle is a rigid layer composed of cross-linked proteins. It gives the cuticle a stiff structure which aids in locomotion and overall body shape. The epicuticle is covered by a thin layer composed of proteins, lipids, and waxes called the envelope. The envelope's primary function is to prevent water loss. Underneath the epicuticle is the procuticle. The procuticle is comprised of a protein-chitin matrix that gives elasticity to the cuticle (Schwarz, 2007). The procuticle is formed by the successive stacking of thin plates with each subsequent layer containing chitin microfibers at a slightly different angle (Meyer, 2005). In some parts of the body, the outer half of the procuticle begins to stratify. Because of this stratification process, the procuticle can also be defined by two sub-layers; the soft, inner endocuticle and the hard, outer exocuticle.

Features of Armet

T. castaneum Armet (TcArmet) is a 168 residue protein containing two domains and a linker region. TcArmet contains a variant of the "KDEL" ER retention signal, suggesting possible function in the endoplasmic reticulum (Figure 1.2). Comparison with other organisms such as *Drosophila melanogaster*, *Acyrthosiphon pisum*, *Mus musculus*, and *Homo sapiens*, reveals many conserved residues throughout the protein, including eight cysteines (Figure 1.2). An evolutionary look at Armet in these five organisms shows that there are two distinct groups;

mammalian Armets and arthropod Armets (Figure 1.3). But any of the insect Armets is nearly as distant from each other as they are to the mammalian Armets (*H. sapiens* and *M. musculus*).

The three-dimensional structure of Armet has only been solved in *H. sapiens* and *M. musculus*. Both structures have been solved using solution nuclear magnetic resonance (NMR) (Hellman et al., 2011) (Hoeski et al., 2010). The crystal structure has also been solved for human Armet (Parkash et al., 2009). As in the case for both proteins, there are two domains connected by a small linker region (Figure 1.4). The N-terminal domain contains four alpha-helices and resembles a saposin-like binding domain (Parkash et al., 2009). The C-terminal domain contains three α-helices and a CKGC disulphide linkage, which is similar to some proteins which have functions in the ER stress response. The differences in these two domains suggested to Parkash et al., 2009 a basis for the bi-functionality of Armet.

Figure 1.2 Alignment of Armet amino acid sequences

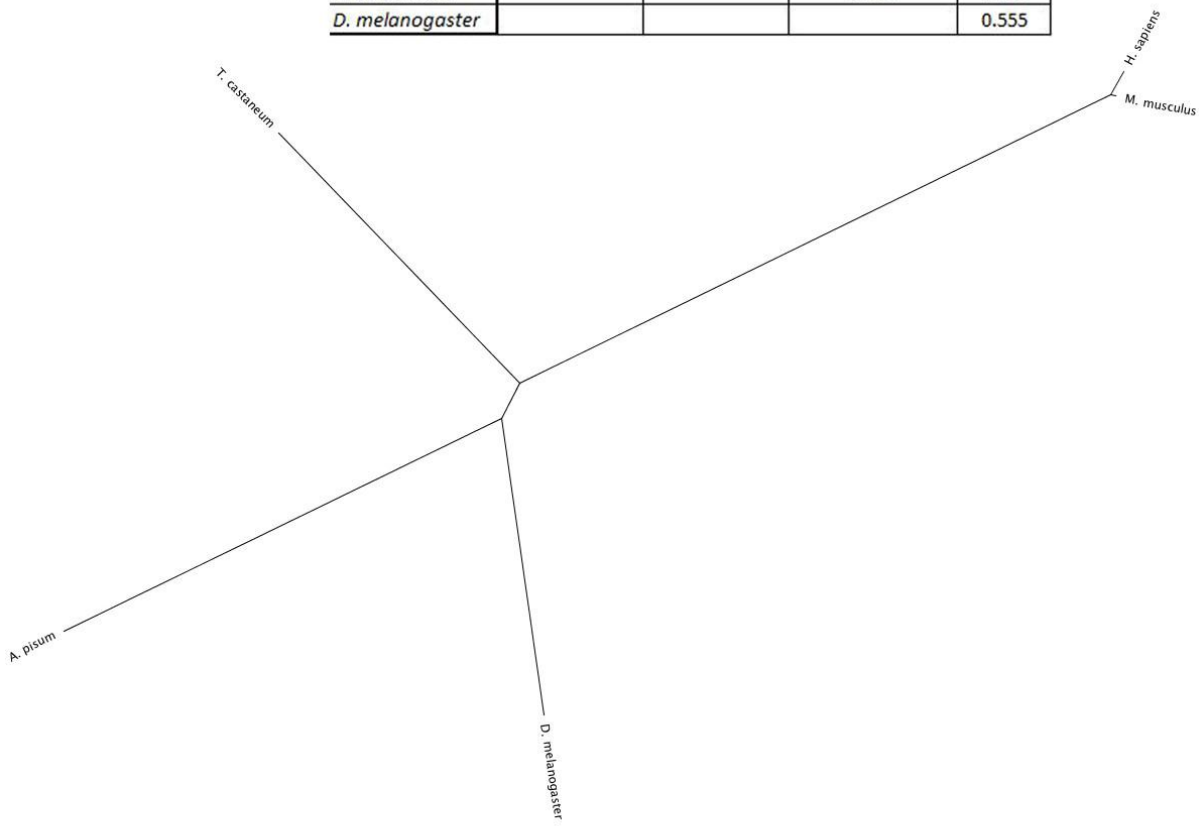
The Armet amino acid sequences for *T. castaneum*, *D. melanogaster*, *A. pisum*, *M. musculus*, and *H. sapiens* were aligned with Clustal Omega (http://www.clustal.org/omega). Consensus symbols used are as follows: fully conserved residue (*), conservation of amino acids with similar properties (:), and conservation of amino acids with weakly similar properties (.). Residues highlighted in yellow constitute signal peptides as determined by Signal P 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Residues highlighted in blue are variants of the canonical "KDEL" ER retention signal. Residues highlighted in red are conserved cysteines (*H. sapiens*: C30-C33, C64-C75, C106-C117, C151-154; Mizobuchi et al., 2007). Residues highlighted in green indicate the linker region for each protein.

```
T. castaneum      MEFQLIFTVLF----ASIVAVNSLKQGECEVCIKVLDKFAASLSD-DVKKDPKLIESKF     54
D. melanogaster   MKTWYMVVVIGFL--ATLAQTSLALKEEDCEVCVKTVRRFADSLDD-STKKDYKQIETAF     57
A. pisum          MDKHILLVCVFFIVFHVFQAQSRTFTEEDCPVCVLTIDKFSKTLEG-E--LNPKNIEEQF     57
M. musculus         MWATRGLAVALALSVLPDSRALRPGDCEVCISYLGRFYQDLKDRDVTFSPATIEEEL     57
H. sapiens        MRRMWATQGLAVALALSVLPGSRALRPGDCEVCISYLGRFYQDLKDRDVTFSPATIENEL     60
                          : .           ::   :* **:  : :*   *.  .   .   **  :

T. castaneum      RDYCKNTR-NKENRFCYYLGGLEESATGILGEMSKPLSWSMPSDKICEKLKKKDAQICEL     113
D. melanogaster   KKFCKAQK-NKEHRFCYYLGGLEESATGILNELSKPLSWSMPAEKICEKLKKKDAQICDL     116
A. pisum          KKYCLSTKIDKEKRLCYYLGGLEDSATGILSEMSKPLSWSMPALKICERLKKMDAQVCDI     117
M. musculus       IKFCREAR-GKENRLCYYIGATDDAATKIINEVSKPLAHHIPVEKICEKLKKKDSQICEL     116
H. sapiens        IKFCREAR-GKENRLCYYIGATDDAATKIINEVSKPLAHHIPVEKICEKLKKKDSQICEL     119
                   .:*   :  **.*:***:*. :::** *: *:****:  :*  ****:*** *:*:*::

T. castaneum      RYDVEIDLKTVDLKKLKVRDLKKIINDWGEDCQGCIEKSEFIQRIEELKHKHTEL>         168
D. melanogaster   RYEKQIDLNSVDLKKLKVRDLKKILNDWDESCDGCLEKGDFIKRIEELKPKYSRSEL>       173
A. pisum          KYDKEIDWKTVNLKKMKVKDLKKILDNWGEICDGCLEKTDYIKRVEELKPSYVKEEL>       174
M. musculus       KYDKQIDLSTVDLKKLRVKELKKILDDWGEMCKGCAEKSDYIRKINELMPKYAPKAASAR     176
H. sapiens        KYDKQIDLSTVDLKKLRVKELKKILDDWGETCKGCAEKSDYIRKINELMPKYAPKAASAR     179
                  :*: :** .:*:***::*::****:::* * *.** ** ::*::::**  .:

T. castaneum          168
D. melanogaster       173
A. pisum              174
M. musculus       TDL> 179
H. sapiens        TDL> 182
```

Figure 1.3 Evolutionary relationship of Armet in multiple organisms

Below are phylogenetic trees for the evolutionary relationships between Armet in the organisms *H. sapiens*, *M. musculus*, *T. castaneum*, *D. melanogaster*, and *A. pisum*. The first tree contains numbers representing branch distances which are calculated by the neighbor-joining tree method, Jukes-Cantor. Numbers closer to zero illustrate a closer evolutionary relationship. The second tree contains bootstrap values to determine confidence in branch arrangements. Numbers closer to 100 are more confident.

	M. musuclus	*T. castaneum*	*D. melanogaster*	*A. pisum*
H. sapiens	0.023	0.721	0.739	0.845
M. musculus		0.721	0.727	0.813
T. castaneum			0.467	0.635
D. melanogaster				0.555



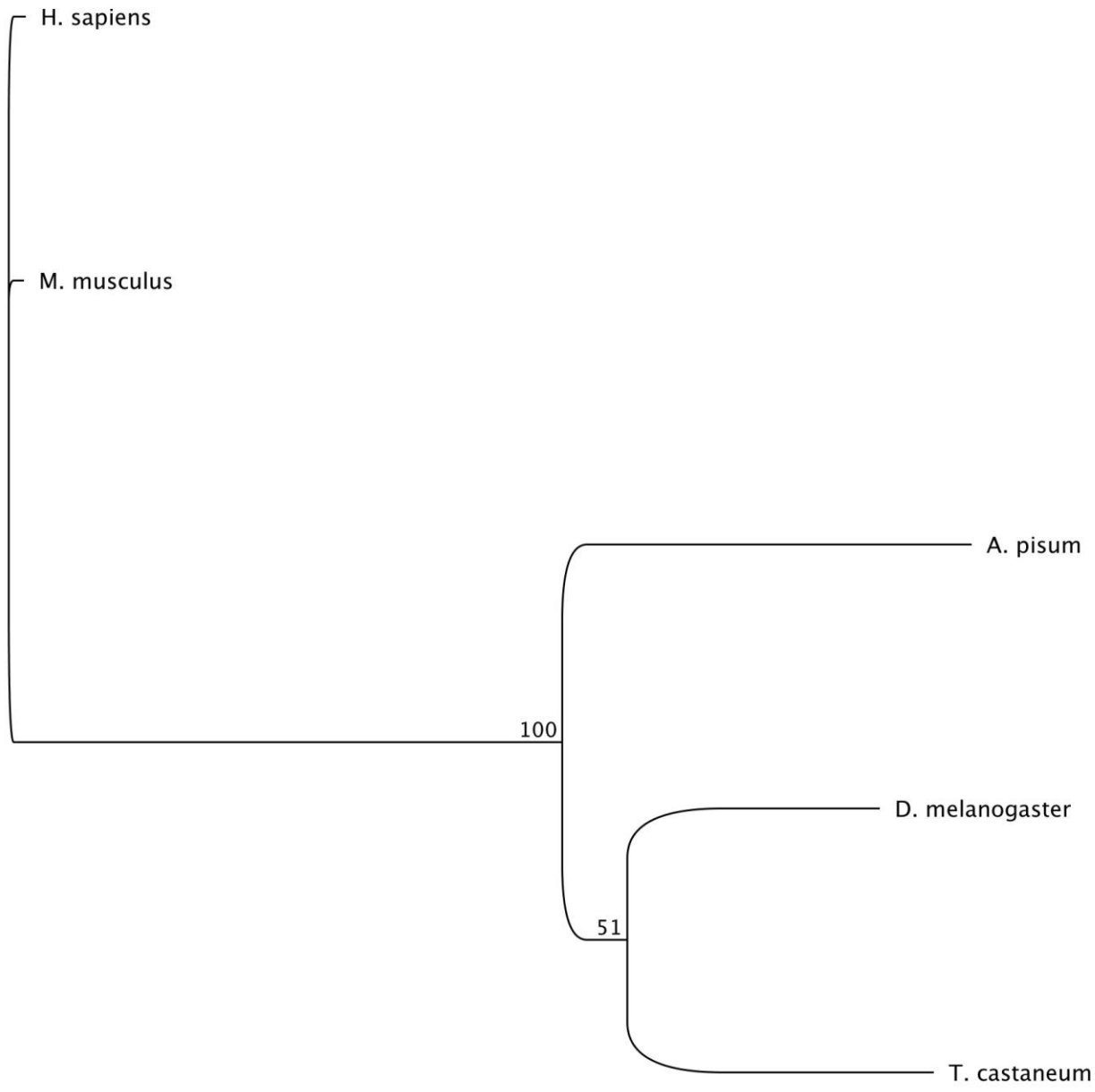
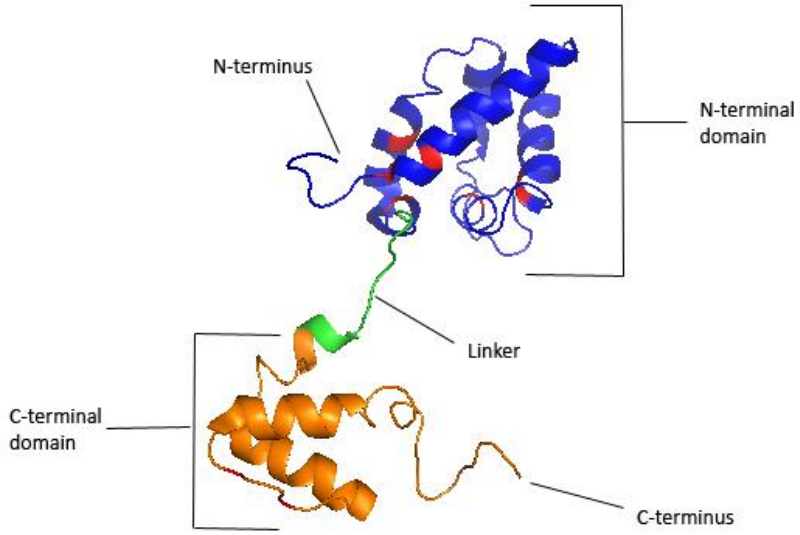

H. sapiens
M. musculus
A. pisum
100
D. melanogaster
51
T. castaneum

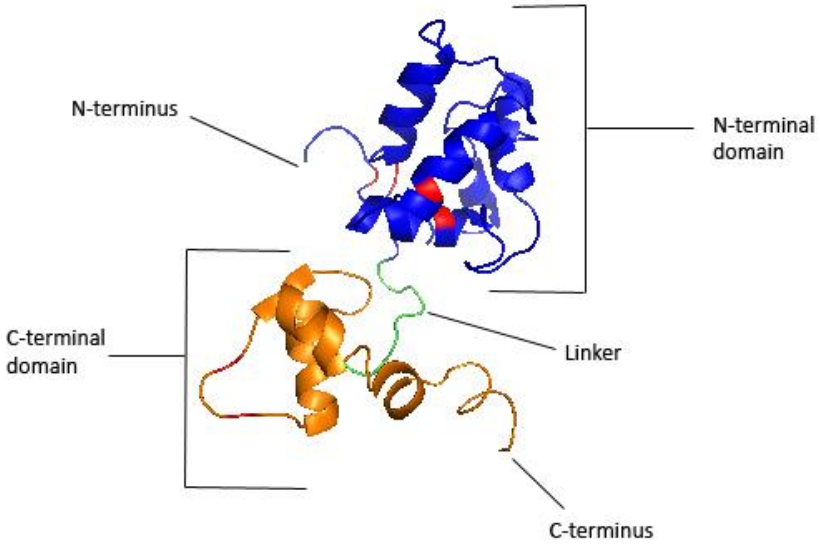
Figure 1.4 Structures of Armet from *H. sapiens* and *M. musculus*

A) Structure of *H. sapiens* Armet as solved by solution NMR (Hellman et al., 2011). B) Structure of *M. musculus* Armet as solved by solution NMR (Hoeski et al., 2010). Cysteine residues are marked in red.

A



B



History of Armet

The Armet transcript was first discovered in human pancreatic ductal adenocarcinomas as a sequence that was often mutated in these tumors. It was originally named arginine-rich protein (ARP) for containing a 55 residue sequence encoding a stretch of arginines (Shridhar et al., 1997). ARP was renamed to arginine rich mutated in early-state tumors (ARMET) when polymorphisms of Armet were found in a variety of early stage tumors and cancers (Shridhar et al., 1996, 1997). It was later found that these polymorphisms were also found in matched normal controls of cancer patients, suggesting that its previous role and annotation may not be accurate (Evron et al., 1997). Nonetheless, Dr. Reeck's lab has retained the name Armet.

Armet was then cloned from human ventral mesencephalic cell line 1 (VMCL1) and found to not contain the 55 residue sequence in the translated product (Petrova et al., 2003). Instead, it was found to be a secretory protein with a signal peptide. Armet goes by another name, mesencephalic astrocyte-derived neurotrophic factor (MANF), since it was shown to possess neurotrophic activity (Petrova et al., 2003).

Apostolou et al. (2008) found Armet to be up-regulated in endoplasmic reticulum (ER) stress and concluded that Armet should be considered a component of the Unfolded Protein Response (UPR). The UPR is a stress response activated upon accumulation of unfolded or misfolded proteins in the ER (Cao et al., 2012). Apostolou et al. (2008) demonstrated that Armet plays a protective role for cells in times of ER stress, preventing cells from undergoing apoptosis. They also found that Armet is both localized in the ER and secreted extracellularly.

Armet and Insect Development

In *D. melanogaster*, insertion of P-elements, in mobilization screens, resulted in deletion of regions of the Armet transcript. Mutant lines homozygous for deleted Armet died as first

instar larvae (Palgi et al., 2009). The lack of Armet led to cell death of neurons and reduced levels of dopamine in embryos. It was also observed that the cuticle of these larvae was defective as it remained permeable to antibodies. This finding was further supported by transmission electron microscopy as it showed severe cuticle disorganization (Palgi et al., 2009). It was hypothesized that this is a consequence of low dopamine levels, which are needed for synthesis of quinones, compounds that cross-link proteins in the cuticle.

Previous research in *T. castaneum* injected with dsArmet RNA has revealed a lethal phenotype in pupae attempting to eclose to adulthood (Heerman, 2012). Characteristics of death include arrest at the late pupal stage upon eclosion and abnormal tanning pigmentation of pupae nearing death and upon death.

Development of T. castaneum Strain PU11

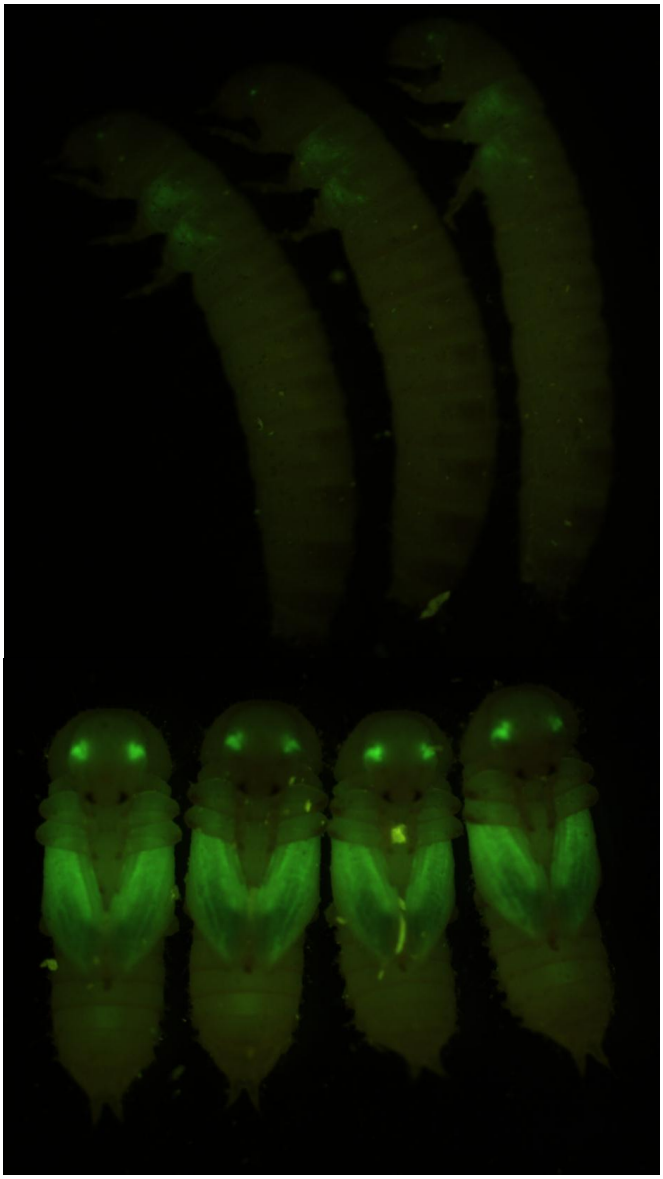
The development of the Tribolium strain PU11, formerly known as Pig-23 (Lorenzen et al., 2003), arose from the need for a universal marker to follow gene transfer. One such marker with an easily visible phenotype is green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. The marker constructed contained an artificial promoter comprised of three binding sites for Pax-6 homodimers in front of a TATA box (3xP3) (Berghammer et al., 1999). The 3xP3-GFP marker was then constructed into a vector based on the piggyBac transposon. Microinjections of these vectors into Tribolium eggs resulted in transgenic lines with a frequency of 60 percent (Berghammer et al., 1999). The major phenotype observed was expression of GFP in photoreceptor cells in the eyes of larvae, pupae, and adults.

Later studies revealed additional, novel GFP expression patterns suggesting that the GFP marker was influenced by chromosomal enhancer sequences near the sites of piggyBac

integration (Lorenzen et al., 2003). One such line, Pig-23 (now called PU11), showed GFP expression in early stages of wing development in the last larval instar (Figure 1.5).

Figure 1.5 GFP fluorescence in uninjected PU11 insects

Fluorescence patterns of GFP in PU11 insects are shown below. Late instar larvae in the top figure show green fluorescence in the thoracic region near leg and wing attachment and in the eyes. Pupae in the bottom figure show fluorescence in the eyes and wings.



Materials and Methods

Insects

Tribolium castaneum strains GA-1 and PU11 were reared at 25˚C or 30˚C. Insects were kept in glass jars with mesh lids on Golden Buffalo Flour (Heartland Mill, Inc.) mixed with 1% yeast.

PU11 insects were viewed on a Leica MZ10 F stereoscope illuminated by a Leica EL6000 external light source through a GFP Plus filter (Leica Microsystems). This filter has an excitation filter at 480/40 nm and a barrier filter at 510 nm.

Time Course of Normal Development

The length of the larval stage and the number of instars, or molts, varies from individual insect to insect. Depending on environmental and physiological conditions, the length of the larval stage can be from 22 to 100 days (Bennett, 2003). Since these numbers offer such a wide range, the timing of development under rearing conditions standard for Tribolium in our lab needed to be determined. A small portion of a large colony was isolated to look for first instar larva. Thirty-five of these larvae were removed and placed in individual microcentrifuge tubes filled with an adequate amount of flour/yeast diet. Individual insects were monitored daily to identify their stage of development.

DNA Isolation

DNA was extracted and purified from larvae. Larvae were homogenized in Ten 9 buffer (pH 9, 50 mM Tris-HCl, 100 mM EDTA, 200 mM NaCl) using a pestle and an electric, rotating

motor (VMR, Cat #47747-370). Additional Ten 9 buffer was added (total volume 500 µL) and the solution was centrifuged for 10 min at 10,000 rpm. The upper aqueous layer was collected and added to 20 µL 20% SDS and 20 µL Proteinase k. The solution was vortexed and incubated at room temperature for 45 min. Equal parts of phenol and chloroform were added to match the current solution volume. The solution was again vortexed and centrifuged for 15 min at 15,000 rpm. The upper aqueous layer was collected and added to 200 µL 3M sodium acetate and 500 µL 70% ethanol in DEPC-treated deionized water. The solution was incubated overnight at -20˚C to further precipitation. After centrifugation for 4 min at 15,000 rpm, the pellet was resuspended in DEPC-treated deionized water. RNase was added to remove any contaminant RNA from solution. Quality and quantity were checked via NanoDrop 2000 (Thermo Scientific).

dsArmet and dsVermilion RNA Synthesis

dsRNAs were prepared using the T-7 PCR method. Genomic DNA isolated from larvae was incorporated with the T-7 promoter via PCR. Primers with the following sequence were used for TcArmet dsRNAs (dsArmet): 5' – TAA TAC GAC TCA CTA TAG GGC CAG TTT ATC AGA CGA CGT GAA – 3' and 5' – TAA TAC GAC TCA CTA TAG GGC TTC AAA TCC CTC ACT TTG AGT TTC – 3' (forward and reverse, respectively). The final product was 356 bp in length, starting at position 110 in the open reading frame and ending at position 405. The primers for the TcVermilion (Accession: NM_001039410.1) dsRNAs (dsVer) were as follows: 5' – TAA TAC GAC TCA CTA TAG GGG AGC AAA TCG CCA AGT CGG – 3' and 5' – TAA TAC GAC TCA CTA TAG GGC CTG GGT TCG TCC CTG TAA – 3' (forward and

reverse, respectively). The final product was 342 bp in length, starting at position 522 in the open reading frame and ending at position 863.

dsRNAs were synthesized from the PCR products using the Ampliscribe™ T7-Flash™ Kit (Epicentre Biotechnologies, Cat #ASF3507). dsRNAs were purified with phenol, chloroform, DNase 1, and RNase A. After centrifugation at 13,000 rpm for 10 min, dsRNAs were extracted with 200 μL chloroform. The solution was centrifuged again at 13,000 rpm for 10 min and to the supernatant 3M sodium acetate and 100% ethanol was added to precipitate the dsRNAs. The solution was centrifuged for 30 min at 13,000 rpm to obtain a pellet of dsRNA. The pellet was washed with 75% ethanol in DEPC-treated deionized water and resuspended in DEPC-treated deionized water. Quality and quantity of the dsRNAs were checked via NanoDrop 2000.

dsRNA Injections

Late instar larvae, with a mass between 3.0 and 4.0 mg, were injected with ~1 μL of dsArmet or dsVer (200 ng/μL). Injections were performed with a pulled glass capillary needle connected to a rubber tube, which was used as a mouth pipette.

RNA Isolation & cDNA Synthesis

To obtain transcript levels of Armet at each life stage for qPCR analysis, RNA was extracted from larvae (mass between 3.0-4.0 mg), pre-pupae, pupae, and adults (3 biological replicates of 5 insects at each stage). The insects were homogenized in 100 μL of TRI Reagent® Solution (Ambion, Cat #AM9738) using a pestle connected to an electric, rotating motor.

Additional TRI Reagent® Solution (for total volume of 1 mL) was added to lyse tissue and cells. After sitting at room temperature for 3 min, 200 µL of chloroform was added to extract RNA. The solution was mixed thoroughly, left to sit for 10 min at room temperature, and centrifuged for 15 min at 12,000 rpm. The organic supernatant was collected and added to 500 µL of isopropanol to precipitate the RNA out of solution. This solution was then washed and purified using the RNeasy Mini Kit (Qiagen, Cat #74104). RNA was eluted in DEPC-treated deionized water.

RNAs were diluted to similar concentrations (100 ng/µL) before proceeding to cDNA synthesis. cDNA was synthesized using the SuperScript® III First-Strand Synthesis System (Invitrogen, Cat #18080-051) for qPCR experiments.

qPCR

Quantitative PCR (qPCR) was conducted to measure transcript levels of Armet, as compared to ribosomal protein RPS6 (Accession: NM_001172390.1), in control and injected insects. Transcript levels were obtained from control insects at the late larval (mass between 3.0-4.0 mg), pre-pupae, pupae, and adult stages. RNA was isolated from three biological replicates of five insects at each stage and cDNA was synthesized from the extracted RNA. Late instar larvae (mass between 3.0 and 4.0 mg) were injected with 200 ng of either dsArmet or dsVer. For both dsArmet and dsVer, three sets of five larvae were injected. RNA was isolated at three different time points after injection; 24h, 48h, and 120h. cDNA was synthesized from the extracted RNA. For Armet, the primers used were: 5’ – CGG TGA ACA GTT TAA AGC AAG G – 3’ and 5’ – TGG ATT CTA TCA ATT TCG GGT CTT - 3’ (forward and reverse, respectively). The primers used for RPS6 were: 5’ – GAA AGG AAA CGC AAG TCA GTT

AG – 3’ and 5’ – ACT TGA GTG TGC TTG CCC TCG TTA – 3’ (forward and reverse, respectively). qPCR experiments were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). To fluorescently label PCR products, iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Cat #172-5121) was used. A melting temperature of 95˚C, annealing temperature of 55˚C, and an extension temperature of 72˚C were used for 40 cycles of PCR.

Statistics

The output data from qPCR experiments are in cycle thresholds (Ct). This value is defined as the number of cycles it takes for the fluorescent signal to cross the threshold or to exceed the background level of fluorescence. The following equation was used to calculate the normalized transcript level of Armet in relation to the transcript level of the “housekeeping gene” RPS6.

$$\textbf{Normalized transcript level} = -[(Ct)_{Armet}-(Ct)_{RPS6}]^2$$

Three biological replicates of five insects were done for each developmental stage or time point after injection, depending on the experiment. The normalized transcript level from all three replicates was averaged and the standard deviation was calculated from the three replicates.

Light Microscopy

Pupae and adult Tribolium were fixed in 4% paraformaldehyde for 24h. Insects were sectioned on a Leitz 1512 rotary microtome (Leica Microsystems) and stained with hematoxylin and eosin. Sections were viewed on a Leica ATC 2000 (Leica Microsystems) and imaged with an InfinityHD lens mount camera (Lumenera Corporation).

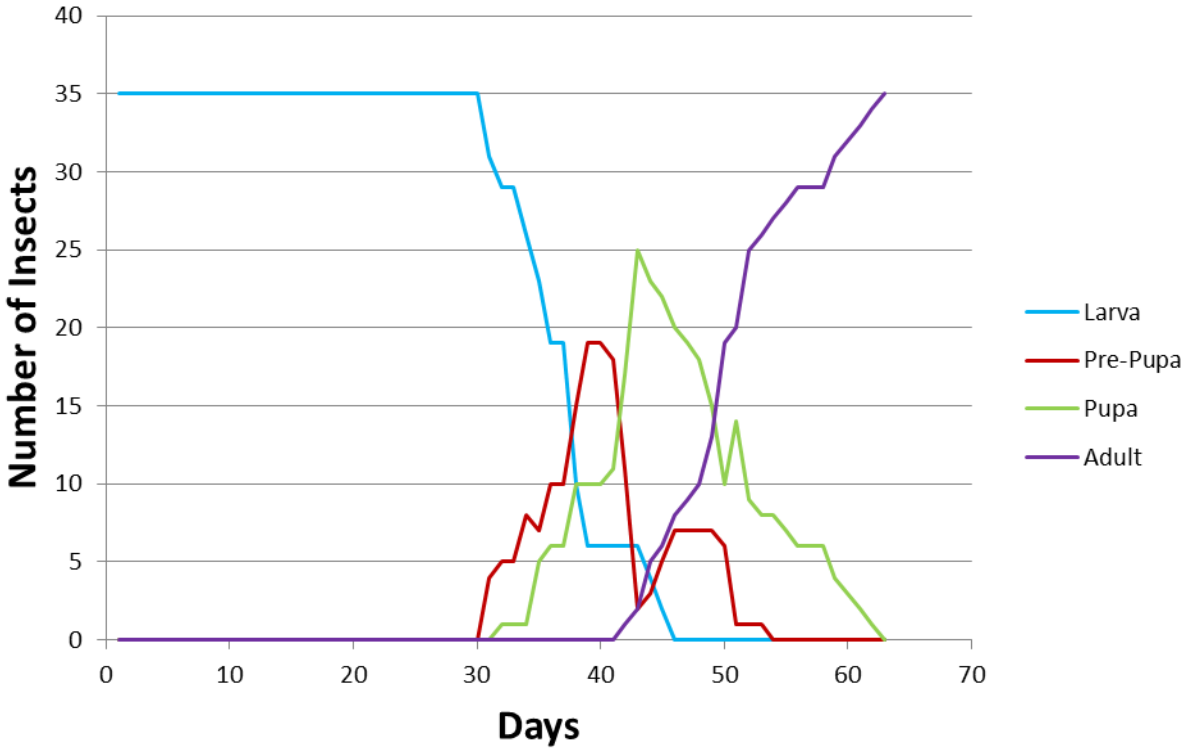
Results

Time Course of Normal Development of Tribolium castaneum

Individual larvae were monitored to determine the length of each developmental stage (Figure 1.6). The average time (in days) for the larval stage was 36.3 ± 4.4 days, pre-pupa was 5.1 ± 1.8 days, and pupa was 8.7 ± 1.5 days. Our hypothesis was that injecting a week before the pre-pupal stage would provide adequate knockdown of the Armet transcript. Therefore, injections would take place approximately 25-30 days into the larval stage. To avoid constantly monitoring larvae after hatching, masses were taken of all larvae from Figure 1.6. The average mass of larvae a week prior to the pre-pupa stage ranged from 3.0-4.0 mg. Therefore, larvae selected for injection had a mass between 3.0-4.0 mg.

Figure 1.6 Progression of development of *T. castaneum*

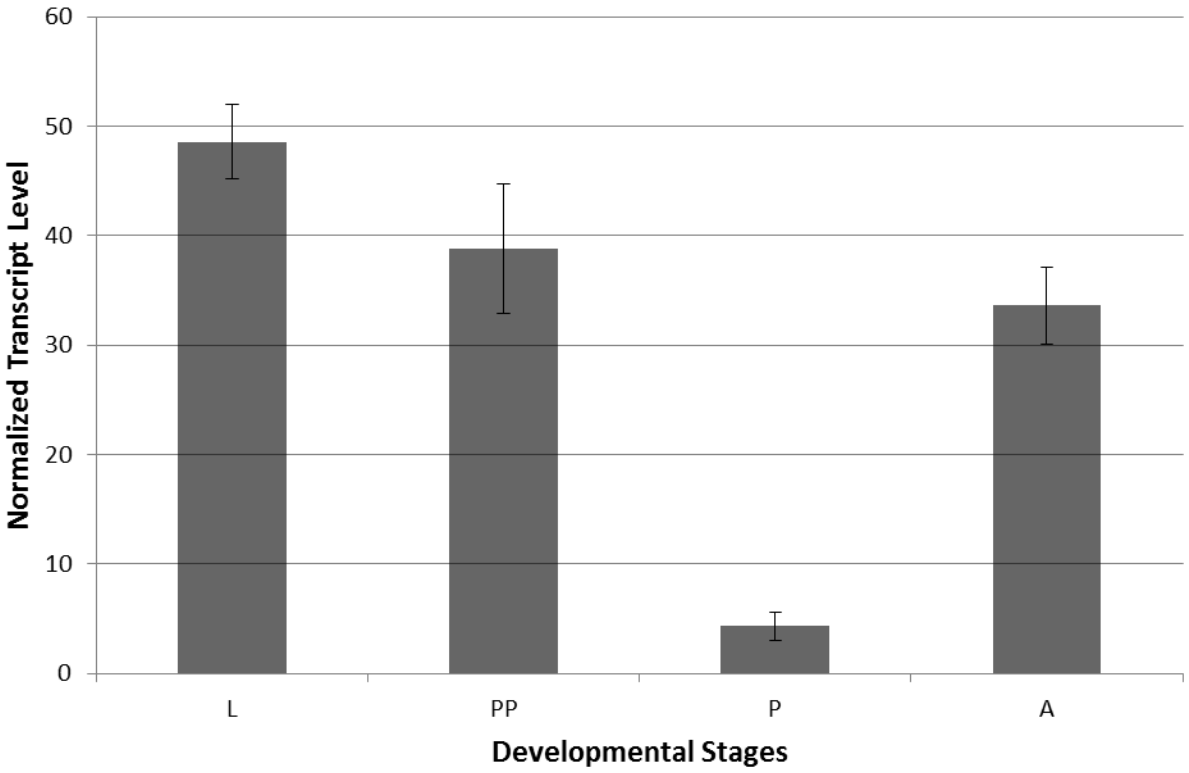
Progression through developmental stages of *T. castaneum* was monitored for larvae, pre-pupae, pupae, and adults. Day zero is defined as the day the insects hatch from their eggs into larvae. The average length (in days) of the larval stage was 36.3 ± 4.4 days, pre-pupa was 5.1 ± 1.8 days, and pupa was 8.7 ± 1.5 days.



Armet Transcript Levels in Developmental Stages

Three biological replicates of five insects were used from the following stages: late instar larva (mass between 3.0-4.0 mg), pre-pupa, pupa, and adult. Pre-pupae were identified by a characteristic banana shape and by lack of movement. Adults were selected based on age, specifically identified by tanning pigmentation. Newly emerged adults show a light tan, or bronze colored cuticle, whereas older adults develop a dark brown cuticle. This change in pigmentation usually occurs about one week into adulthood. The adults with darker cuticles were selected for qPCR analysis. Total RNA was extracted and used as a template for cDNA synthesis. Armet transcript levels were normalized to the "housekeeping gene" ribosomal protein S6 (RPS6). As shown in Figure 1.7, the larval, pre-pupal, and adult stages showed relatively high amounts of the Armet transcript, with the larval stage having the highest level. The pupal stage had the lowest transcript level, over a 10-fold decrease compared to the total transcript level in the larval stage. These results suggest the best stage for transcript knockdown via RNAi would be in the late instar larval stage.

Figure 1.7 Armet transcript levels at different stages of development for *T. castaneum*
qPCR transcript levels of Armet at larval (L), pre-pupal (PP), pupal (P), and adult (A) stages. Larvae with a mass between 3.0-4.0 mg were used. Pre-pupae were identified by a curved, banana-shaped body. Adults were selected by age based on tanning colorization. Adults (1-2 weeks) having a dark brown cuticle were selected for analysis. Three biological replicates of five insects were profiled. Armet qPCR signals were normalized against qPCR signals for ribosomal protein RPS6. Error bars represent standard deviations.



Armet Transcript Knockdown via RNAi

To examine the efficiency of RNAi-mediated knockdown of the Armet transcript, qPCR was used. Late instar larvae were injected with either dsArmet RNA or dsVer RNA. Total RNA was extracted at three different time points after injection; 24h, 48h, and 120h. Three biological

replicates of five insects were employed at each time point. Armet transcript levels were normalized against the RPS6 transcript. As seen in Figures 1.8 and 1.9, Armet transcript levels were significantly reduced in insects injected with dsArmet RNA compared to those injected with dsVer RNA. Transcript levels of Armet were greatly reduced by 24h and were nearly absent 120h after injection, an approximately 27-fold reduction from transcript levels in un-injected larvae.

Late instar larvae (mass between 3.0 and 4.0 mg) were injected with dsArmet RNA or dsVermilion (dsVer). Approximately 40 larvae were injected with dsArmet RNA or dsVer RNA and monitored as they progressed through their respective developmental stages (Figures 1.11 & 1.12). Day zero is defined as the day of injection. Larvae injected with dsVer RNA (Figure 1.10) had a much higher survival rate than dsArmet RNA injected larvae. For dsArmet RNA injected insects, pupation occurred for most larvae between days 10 through 20, and around day 18, a large increase in deaths occurred. This increase corresponded with a sharp decline in the number of pupae. Pupae that are dying begin to show a dark brown, tanning phenotype in the wings (Figures 1.13 & 1.14). Upon death, this dark brown color extends to the rest of the pupal body. This relationship points to a possible fatal phenotype occurring as the pupa attempts to eclose to the adult stage. Also supporting this theory is the fact that some pupae died during the process of eclosion (Figure 1.15). Here, the pupae looked to be in an intermediate stage between pupa and adult when death occurred. Finally, we see a small percentage of insects (1 out of 30) dying at the pre-pupa stage (Figure 1.16). Characteristics of death at this stage include a shriveled body and dark brown colorization.

Figure 1.8 Armet transcript levels in knockdown insects

qPCR transcript levels of Armet for insects injected with dsArmet RNA or dsVer RNA. Late instar larva (mass between 3.0-4.0 mg) were injected with either dsArmet RNA or dsVer RNA. RNA was isolated 24h, 48h, and 120h from injected insects (3 replicates of 5 insects). cDNA was synthesized from extracted RNA. Armet qPCR signals were normalized against qPCR signals for ribosomal protein RPS6. Error bars represent standard deviation. The red, dashed line indicates the transcript level of Armet control insects that were not injected (Figure 1.7).

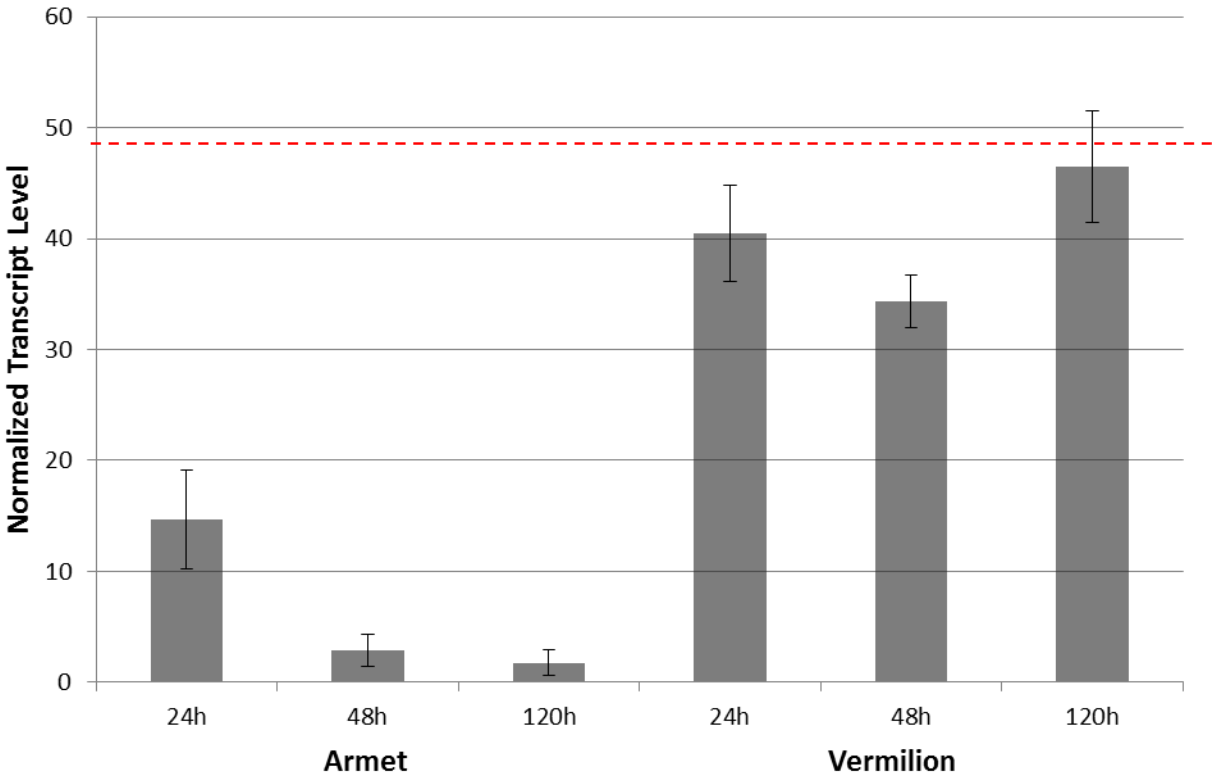


Figure 1.9 Time course of Armet transcript levels in dsRNA injected insects

qPCR transcript levels of Armet transcript in insects injected with dsArmet RNA or dsVer RNA over time. Armet qPCR signals were normalized against qPCR signals for ribosomal protein RPS6. (Data taken from Figure 1.8)

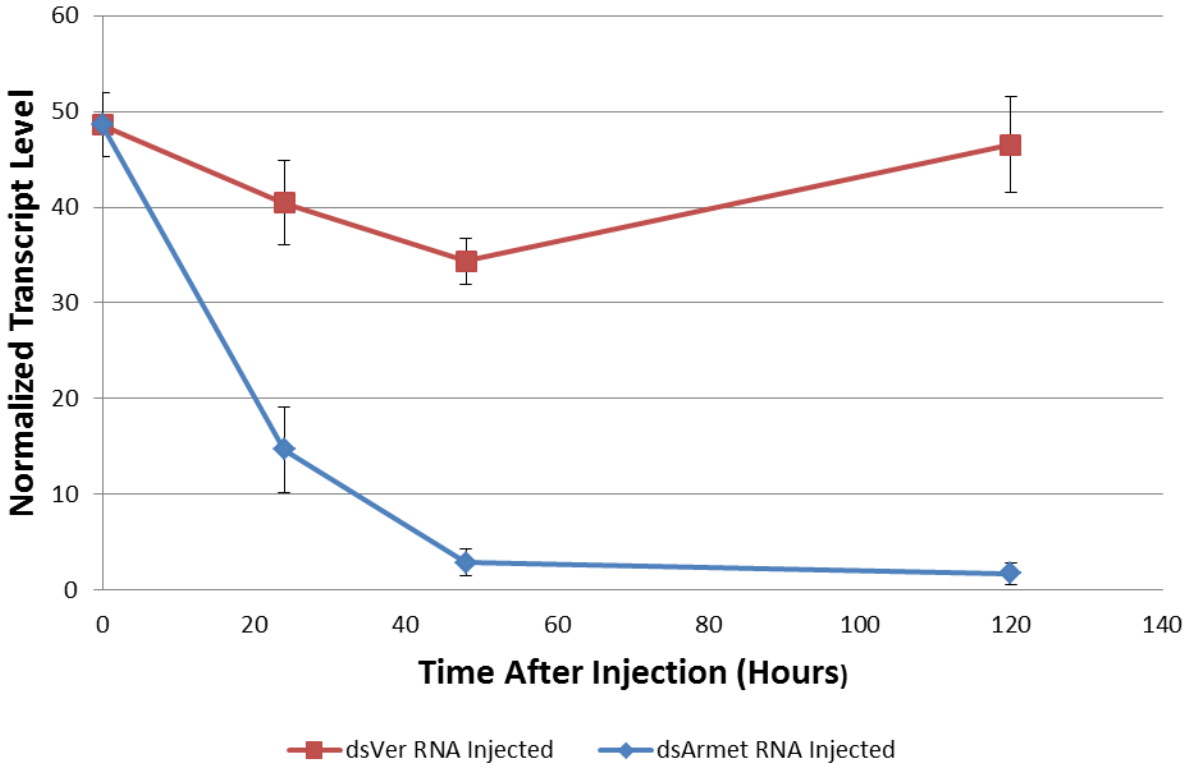


Figure 1.10 Pigmentless eye phenotype in dsVer RNA knockdown insects

On the right, larvae injected with dsVer RNA exhibit the characteristic white, pigmentless eye color in the pupal stage. On the left is an uninjected pupa showing wild-type eye color.

Figure 1.11 Progression of development in dsVer RNA knockdown insects

Progression through the stages of development was monitored after injection of dsVer RNA. Late instar larvae with a mass between 3.0-4.0 mg were injected with ~1 µL dsVer RNA (200 ng/µL). Day 0 is defined as the day of injection. (Data re-plotted from Heerman, 2014).

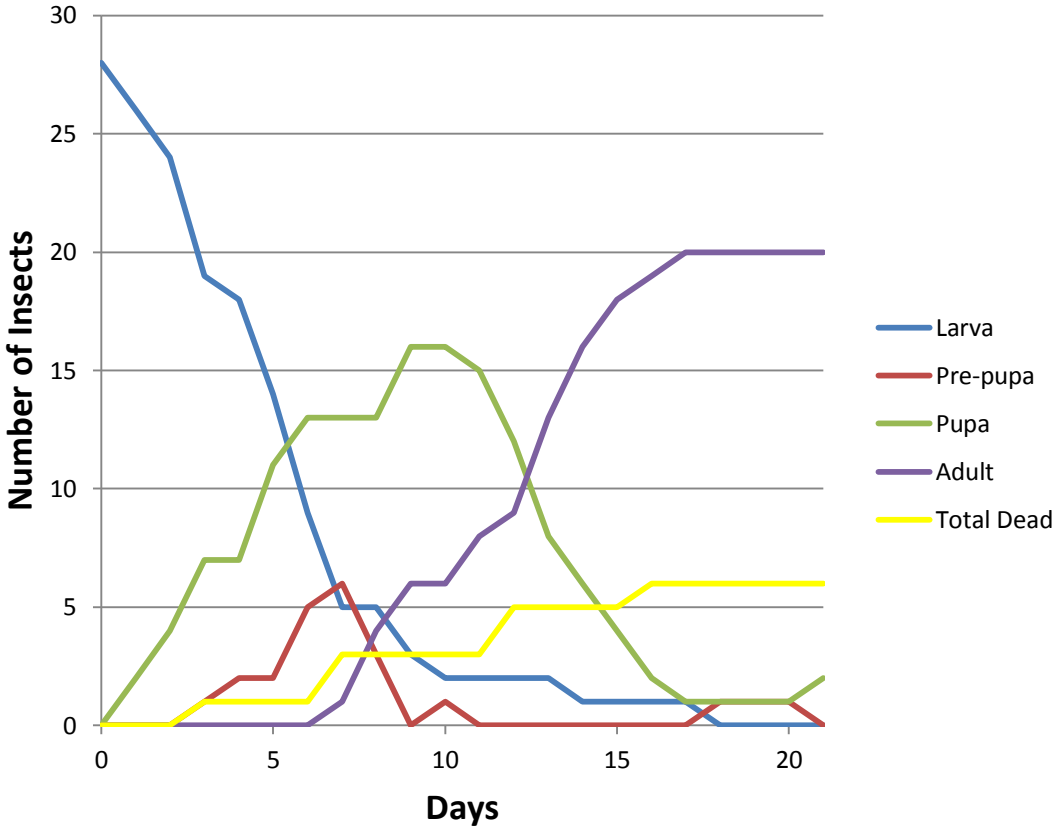


Figure 1.12 Progression of development in dsArmet RNA knockdown insects

Progression through the stages of development was monitored after injection with dsArmet RNA. Late instar larvae with a mass between 3.0-4.0 mg were injected with dsArmet RNA (200 ng/μL). Day 0 is defined as the day of injection.

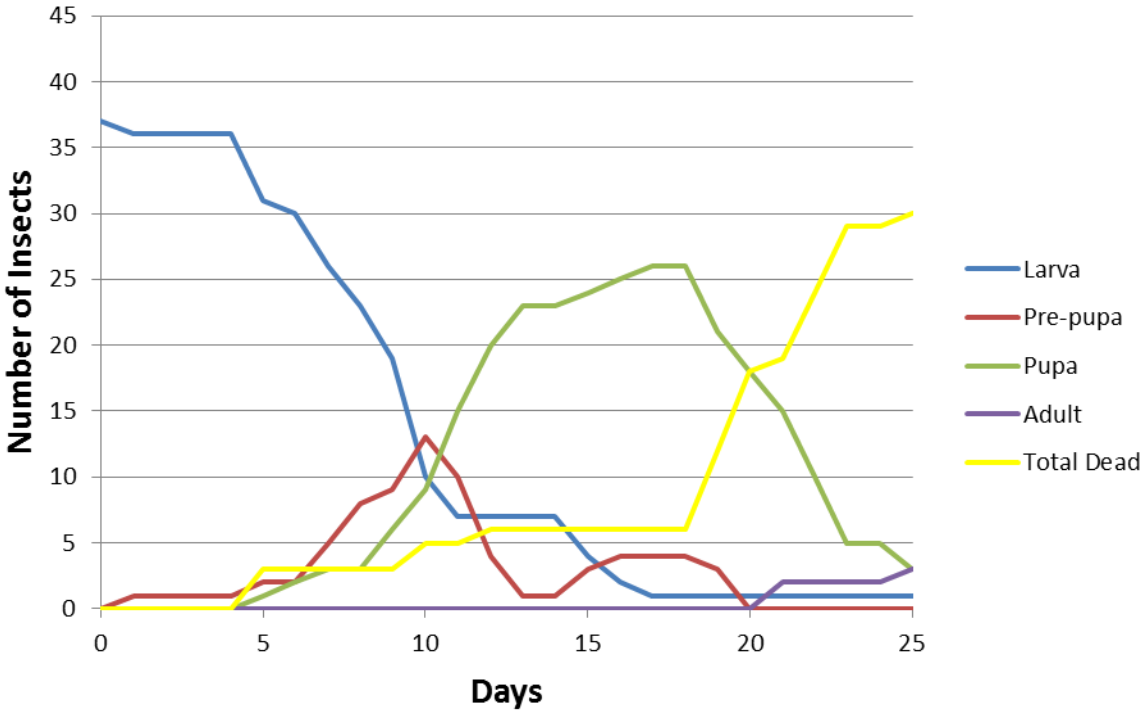


Figure 1.13 Fatal phenotypes observed in Armet knockdown insects

Tribolium injected with dsArmet RNA display abnormal tanning phenotypes when nearing death. The three pupae on the left exhibit the tanning characteristics, found mainly in the wings, of those nearing death. The pupa on the far right has died and has the tanning characteristics associated with the lethal phenotype, specifically the dark color in the wings and brown color of the body.

Figure 1.14 Additional photos of fatal phenotypes observed in Armet knockdown insects

Pupae exhibiting fatal phenotypes show characteristics of abnormal tanning in the wings and body. A) Dead pupa showing dark tanning on the body but no tanning in the wings. B) Pupa close to death and beginning to display the dark tanning on its wings and body. C) Pupa showing unusual tanning near wing attachment to the body as indicated by the arrow. D) Pupa showing darker tanning throughout body.

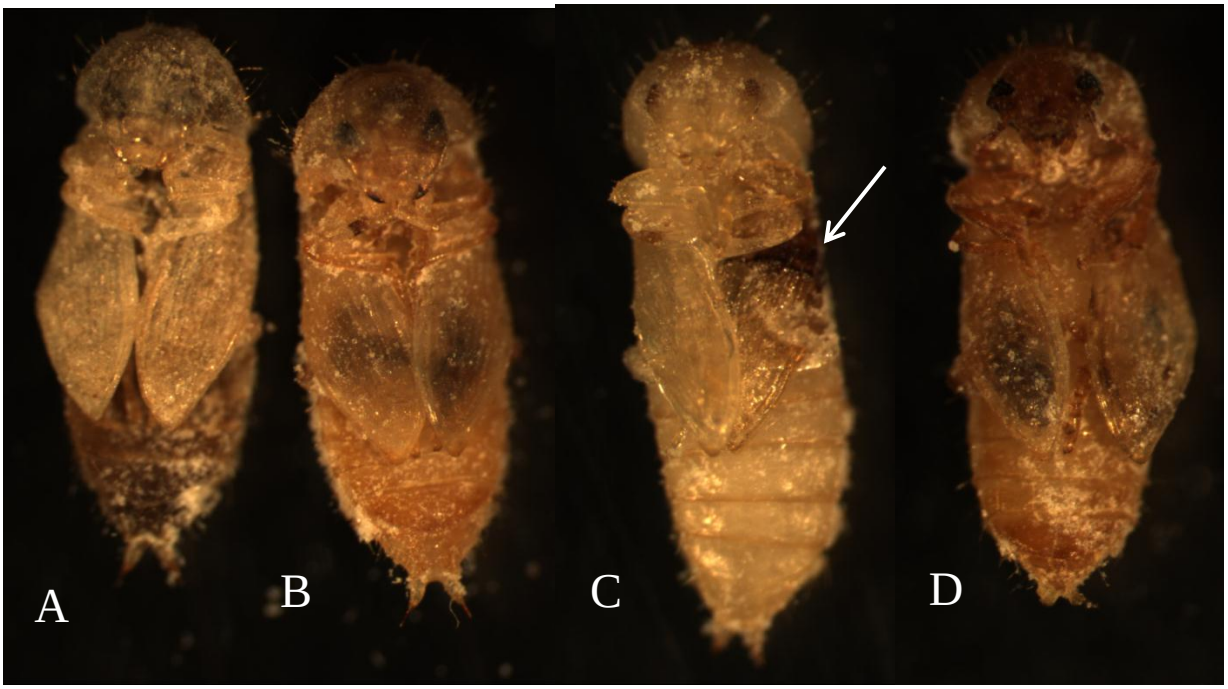


Figure 1.15 Death during eclosion in Armet knockdown insects

In the instances that some pupae were able to start eclosion, death occurred during eclosion.

These insects died in an intermediate phase between pupa and adult.

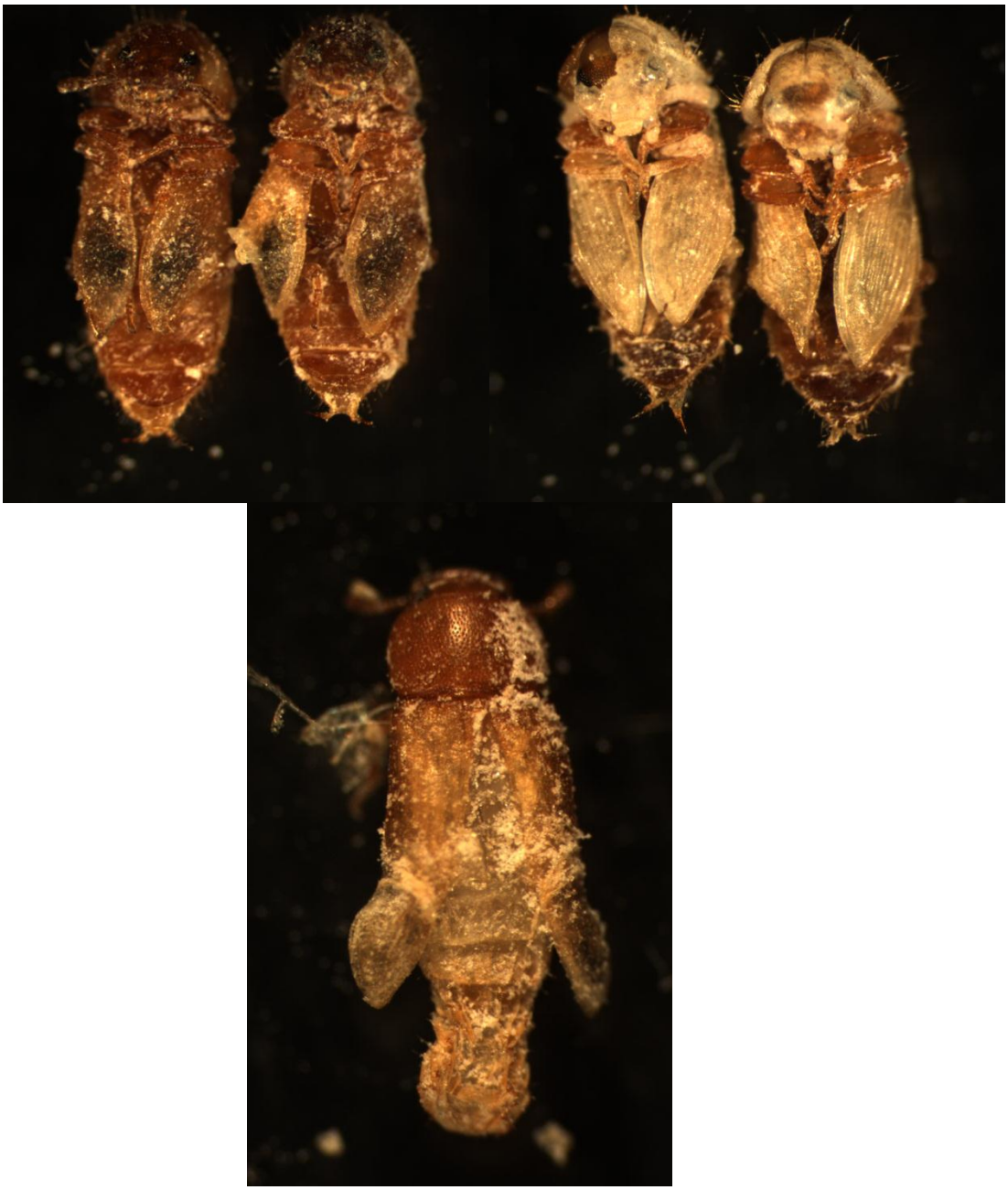


Figure 1.16 Pre-pupa fatal phenotype in Armet knockdown insects

Another phenotype observed occurred when the insects could not pupate from the pre-pupal stage. Characteristics of this phenotype include a withered body and occasional tanning of the body. This was a rare phenotype (roughly 1% frequency).

Armet Transcript Knockdown in T. castaneum Strain PU11

The PU11 strain of *T. castaneum* provides an indicator of developmental progress. These insects contain a GFP-encoding gene that fluoresces in the thoracic region of larvae with early development of wings in the final instar. This lines up very well with the time table previously established for injection of dsArmet RNA, but provides more precision in determining stage of development for timing our dsRNA injections.

PU11 larvae that had begun to fluoresce within the previous 12h were selected for injection. As with the GA-1 strain, larvae were injected with either dsArmet RNA or dsVer RNA. Comparing the progression curves of dsArmet RNA-injected insects seen in Figure 1.12 to the equivalent curve for PU11 (Figure 1.17), we see a very similar trend between the two strains. PU11 pupae show the same characteristics of the lethal phenotype as did the GA-1 strain, namely the tanning colorization in the wings and body (Figure 1.18). Deaths were also observed during eclosion and in the pre-pupal stage (Figures 1.19 & 1.20, respectively). This is similar to the results found in the GA-1 strain. We also observed the fluorescence pattern of GFP in knockdown insects. As illustrated in Figure 1.18, knockdown insects show a decreased intensity of GFP signal.

Figure 1.17 Progression of development in PU11 Armet knockdown insects

Progression through the stages of development was monitored after injection of dsArmet RNA. Larvae that had recently showed fluorescence of EGFP in the thoracic region were selected for injection with 200 ng dsArmet RNA. Day 0 is defined as the day of injection.

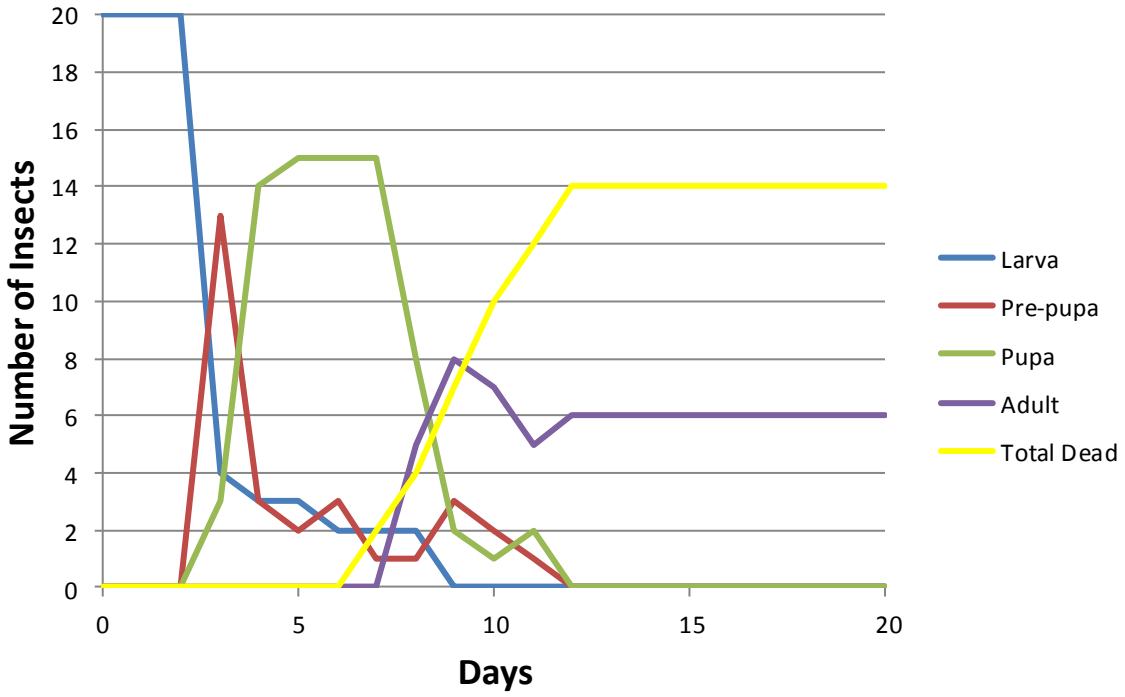


Figure 1.18 Armet knockdowns in the PU11 strain

An array of fatal phenotypes showing similar characteristics to knockdowns in the GA-1 Tribolium strain is shown below. A) Pupae 1 & 3 are nearing death and are beginning to exhibit a tanning phenotype in the wings. Pupa 2 is dead and showed the dark tanning phenotype in its entire body. Pupa 4 is an early pupa yet to show the very dark tanning in the wings and body. B) The same insects shown under UV light. The expression of GFP in pupa 2 is weaker and scattered as compared to pupa 4.

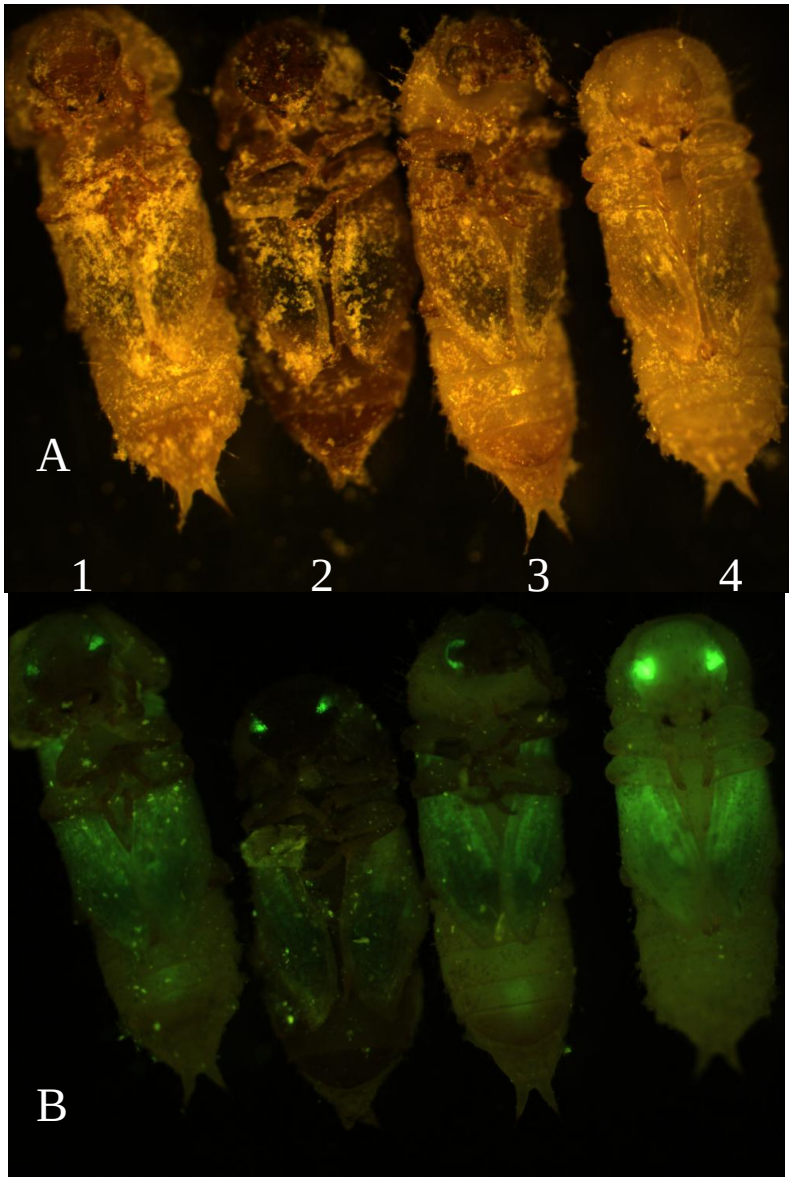


Figure 1.19 Fatal phenotypes during eclosion in PU11 Armet knockdowns

Fatality is also observed when pupae attempt to eclose to the adult stage. A) Ventral view. B) Dorsal view. GFP fluorescence is not shown because it is very faint and difficult to see.

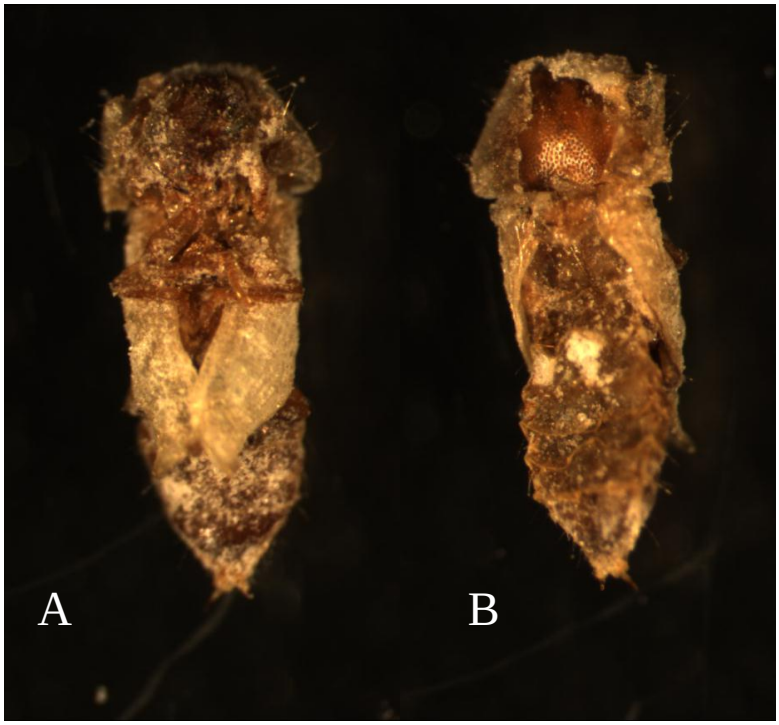


Figure 1.20 Pre-pupal fatal phenotype in PU11 Armet knockdowns

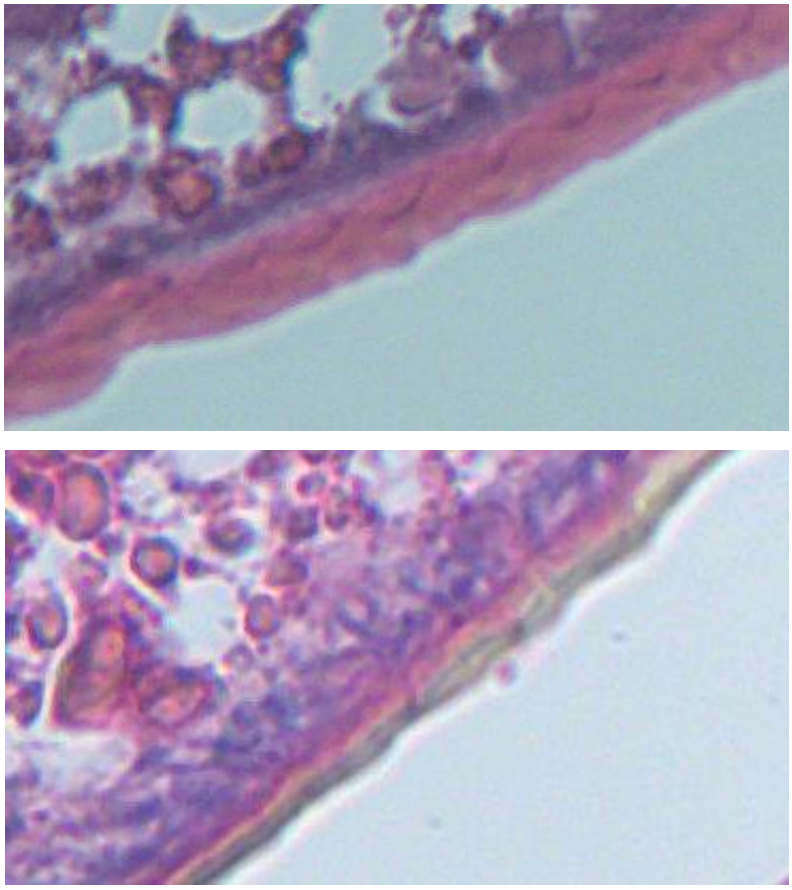
As seen in the GA-1 strain, a small percentage of insects displayed a fatal phenotype at the pre-pupal stage. Characteristics of this phenotype include a shriveled body and slight colorization.

Light Microscopy of Cuticle Organization

To observe possible perturbations in cuticle organization, control and injected pupae were sectioned with a rotary microtome and stained with hematoxylin and eosin. Initial findings (Figure 1.21) suggested differences in both disorder in exocuticle and epicuticle in dsArmet RNA-injected pupae as compared to uninjected control pupae. The exocuticle for dsArmet RNA knockdown pupae was unstained and the entire cuticle narrower than in the uninjected insect.

Figure 1.21 Light microscopy of cuticle in control and injected pupae

The top panel shows the cuticle of an uninjected pupa in the thoracic exoskeleton. The bottom panel shows the cuticle of a pupa injected with dsArmet RNA in the thoracic exoskeleton. The level of magnification is 40x.



Discussion

Armet Transcript and Protein Levels

The Armet transcript is found in all developmental stages in *T. castaneum*. The highest levels of the Armet transcript are found in the larval, pre-pupal, and adult stages. There is a large reduction in the amount of Armet transcript in the pupal stage. It is possible that the insect produces a large amount of Armet protein in the larval stage to have a reserve ready in the pupal stage as it prepares to eclose to adulthood. As described by Chaudhari et al. (2011), cuticle synthesis is a very tightly regulated process involving many secreted proteins. One might presume that such a process invokes a great deal of ER stress in cells. The presence of high amounts of Armet might protect the epidermal cells from apoptosis if it functions as a component of the unfolded protein response in insects as it does in human and mouse (Apostolou et al., 2008). I hypothesize that by injecting dsArmet RNA in final instar larvae and knocking down Armet transcript levels, I am preventing the insect from producing enough Armet protein for the pupal stage and eclosion.

Armet Disrupts Cuticle Formation

Knockdown of the Armet transcript resulted in death as pupae attempted to eclose into adults. Some deaths also occurred at the pre-pupal stage. Both transitional processes (pre-pupa → pupa; pupa → adult) are complex and rely on secretion of many proteins to form the cuticle (Chaudhari et al., 2011; Arakane et al., 2008). Perturbations of cuticle color and organization in dsArmet RNA-injected insects suggest that loss of the Armet protein disrupts cuticle formation. Previous studies in *D. melanogaster* suggest that reduction in dopamine levels, which are needed for secretion of quinones, may be the cause of death in embryos lacking Armet (Palgi et al.,

2009), but cuticle formation could also be disrupted because Armet is not present in the ER to meditate ER stress as a result from unfolded or misfolded proteins.

PU11 is Precise in Determining Developmental Stage

The Tribolium strain PU11 provides a precise means of determining the stage of development for dsRNA injections. Fluorescence in larvae occurs during the final instar before pupation. Results from knockdowns in PU11 insects compared to knockdowns in the GA-1 strain remained fairly consistent. However, there were a higher number of insects that survived eclosion into adulthood in the PU11 strain than in the GA-1 strain. An explanation for this observation is that fluorescence in late instar larvae occurs just a few days after the last possible time for injection to obtain maximum knockout efficiency of the Armet transcript. It is possible that there is already sufficient Armet protein made to facilitate eclosion into adulthood by the time the larvae starts to fluoresce. The overall results however, suggest that the PU11 strain is a reliable method for determining the precise time point in which to inject.

Conclusion

The Armet transcript is expressed throughout development in the model organism *Tribolium castaneum*, with the highest level of Armet transcript found in the larval stage. Injections of dsArmet RNA in late instar larvae significantly knocks down the transcript level for Armet after 24h, with nearly all of the Armet transcript depleted after 48h. Knockdown of the Armet transcript in late instar larvae results in a lethal phenotype occurring as the insects attempt to eclose from the pupal stage into adulthood. Characteristics of death include abnormal tanning pigmentation in the wings and body of pupae before eclosion. Similar phenotypes were

observed in some pre-pupae. An initial investigation of this phenotype by light microscopy revealed disorganization in the cuticle of Tribolium when the Armet transcript is depleted. The cause of death may be a result of the build-up of unfolded or misfolded proteins in the ER, causing ER stress. Without the Armet protein assisting with the UPR, the proper molecules and cross-linking components would not be properly secreted to form the cuticle.

Injections of dsArmet RNA in the Tribolium strain PU11 gave similar death curves as seen in the wild-type GA-1 strain. Therefore, GFP expression in the body of late instar larvae gives an accurate marker for the proper time point for injection. The PU11 strain may be a more reliable marker of development for future studies using RNAi.

There are many possible theoretical uses for these results in the grain industry. One method would be to incorporate a region of the Armet gene into the genome of wheat so dsArmet RNA would be synthesized in the endosperm of wheat kernels. Presumably, Tribolium feeding on these genetically modified grains would die as a result of ingestion of dsArmet RNA-rich endosperm. A possible problem arises if there is significant sequence similarity between the Armet coding sequence of Tribolium with the Armet coding sequences of humans and common livestock. If a region of the Tribolium Armet coding sequence selected for insertion into the wheat genome had near perfect complementarity to human and livestock Armet coding sequences, then dsRNA fragments resulting from the Dicer enzyme in the RNAi pathway could have a negative effect on the humans handling the grain and the livestock which feed on it. A nucleotide alignment of the Armet coding sequence for *T. castaneum*, *H. sapiens*, *Equus callabus* (horse), *Bos taurus* (cow), and *Sus scrofa* (pig) is shown in Figure 1.22. A possible region of the Tribolium coding sequence for insertion into the wheat genome is highlighted. Picking any fragment of 20-21 nucleotides in this region will never result in more than half of

the nucleotides in all five species being completely conserved. For the RNAi pathway to successfully silence the target gene, the dsRNA fragment used as a template has to have near perfect complementarity to the mRNA it is targeting (Wilson et al., 2013). Therefore, inserting a region of the Tribolium Armet coding sequence into grain products has the potential to be a solution to the problem of Tribolium infestation.

Figure 1.22 Alignment of Armet coding sequences in Tribolium, human, and livestock

A sequence alignment of the Armet coding sequence for *T. castaneum*, *H. sapiens*, *E. callabus*, *B. taurus*, and *S. scrofa*. The highlighted section is a region that could possibly be inserted into the genome of wheat or other grain product. Any 20-21 nt fragment within this region will never have more than half perfect complementarity. Below is the distance matrix for these five species, calculated by the Jukes-Cantor neighbor-joining tree model.

```
Tc          ATGGAATTTCA-----ATTAATCTTCACTGTTCTATTCGCA---AGCATCGTCGCG       48
Hs      ATGAGGAGGATGTGGGCCACGCAGGGGCTGGCGGTGGCGCTGGCTCTGAGCGTGCTGCCG       60
Ec              ATGTGGGCCACGCACGGGCTGGCGGTGGCGGTGGCGTTGAGCGTGCTGCCG       51
Bt              ATGTGGGCCACCCACGGGCTGGCCGTGGCGCTGGCTCTGAGCGTGCTGCCG       51
Ss              ATGTGGTTCACTCACGGGCTGGCAGTGGCGCTGGCTCTGAGCGTGCTGCCA       51
                ** *               *  * **     * **    *** *  *  *

Tc      GTG---AACAGTTTAAAGCAAGGAGAATGCGAAGTCTGTATCAAAGTCTTGGACAAATTC       105
Hs      GGCAGCCGGGCGCTGCGGCCGGGCGACTGCGAAGTTTGTATTTCTTATCTGGGAAGATTT       120
Ec      GGCAGTCGGTCGCTGCGACCCGGCGACTGCGAAGTGTGTATTTCCTACCTGGGAAGGTTT       111
Bt      GCCAGCCGGGCGCTGCGACAGGGTGACTGCGAAGTTTGTATTTCTTATCTGGGAAGATTT       111
Ss      GCAAGCCGGGCCCTACGGCCGGGCGACTGTGAAGTTTGTATTTCTTACCTGGGAAGATTT       111
        *            *    *  ** ** ** ***** *****        ***  *  **

Tc      GCGGCCAGTTTATCAG---ACGACGTGAAAAAAGACCCGAAATTGATAGAATCCAAATTT       162
Hs      TACCAGGACCTCAAAGACAGAGATGTCACATTCTCACCAGCCACTATTGAAAACGAACTT       180
Ec      TACCAGGACCTCAAAAACAGAGATGTCACATTCTCACCAGCTTCTGTTGAAAAAGAACTT       171
Bt      TACCAGGACCTCAAAGACAGAGATGTCACATTCTCTCCAGCCTCTATTGAAAAAGAACTT       171
Ss      TATCAGGACCTCAAAGACAGAGATGTCACATTCTCACCAGCCTCTATTGAAAAAGAACTT       171
                 *   *      ** ** * *      **        * ***    ** **

Tc      CGGGACTATTGTAAAAACACGAGAAACAAGGAGAATCGATTTTGTTACTACTTGGGGGGC       222
Hs      ATAAAGTTCTGCCGGGAAGCAAGAGGCAAAGAGAATCGGTTGTGCTACTATATCGGGGCC       240
Ec      ACAAAGTTCTGCCGTGAAGCAAGAGGCAAAGAGAATCGGTTGTGCTACTACATTGGGGCC       231
Bt      ATAAAGTTCTGCCGTGAAGCAAGAGGCAAAGAGAATCGGTTGTGCTACTACATTGGGGCC       231
Ss      ACAAAGTTCTGCCGTGAAGCCAGAGGCAAAGAGAATCGGTTGTGCTACTACATTGGGGCC       231
            * *  **     *  * ***  *** ******** ** ** *****  * **** *

Tc      CTTGAGGAAAGTGCCACTGGTATTTTGGGCGAAATGTCCAAACCGCTTTCATGGTCAATG       282
Hs      ACAGATGATGCAGCCACCAAAATCATCAATGAGGTATCAAAGCCTCTGGCCCACCACATC       300
Ec      ACAGATGATGCAGCCACCAAGATCATCAATGAGGTGTCAAAGCCCCTGGCCCACCACATC       291
Bt      ACAGAGGATGCAGCCACCAAGATCATCAACGAGGTGTCCAAGCCCCTGTCCCACCACATC       291
Ss      ACAGATGATGCAGCCACCAAGATCATCAATGAGGTGTCGAAGCCCCTGGCCCACCACATC       291
           ** **    *****    **  *    **  * ** ** ** **  *       **

Tc      CCATCGGACAAAATCTGCGAAAAATTGAAGAAAAAAGACGCCCAAATCTGCGAATTGCGC       342
Hs      CCTGTGGAGAAGATCTGTGAGAAGCTTAAGAAGAAGGACAGCCAGATATGTGAGCTTAAG       360
Ec      CCTGTGGAGAAGATCTGTGAGAAGCTCAAGAAGAAGGACAGCCAGATCTGTGAGCTAAAG       351
Bt      CCTGTGGAGAAGATCTGTGAGAAGCTCAAGAAGAAAGACAGTCAGATCTGTGAACTAAAG       351
Ss      CCTGTGGAGAAGATCTGTGAGAAGCTCAAGAAGAAGGACAGCCAGATCTGTGAGCTAAAG       351
        **   *** ** ***** ** **  * ***** ** ***   ** ** ** **  *

Tc      TACGACGTCGAAATCGATTTAAAGACAGTTGATTTGAAGAAACTCAAAGTGAGGGATTTG       402
Hs      TATGACAAGCAGATCGACCTGAGCACAGTGGACCTGAAGAAGCTCCGAGTTAAAGAGCTG       420
Ec      TATGACAAGCAGATCGACCTGAGCACAGTGGACCTGAAGAAGCTCCGAGTTAAAGAGCTA       411
Bt      TATGACAAACAGATCGACCTGAGCACAGTGGACCTGAAGAAGCTCCGAGTTAAAGAGCTA       411
Ss      TATGACAAGCAGATCGACCTGAGCACAGTGGACCTGAAGAAGCTCCGAGTTAAAGAGCTA       411
        ** ***    * *****  * *  ***** **  ******* ***  *** * **  *

Tc      AAGAAGATTATCAACGACTGGGGCGAAGACTGCCAAGGGTGCATCGAAAAAAGCGAGTTC       462
Hs      AAGAAGATTCTGGATGACTGGGGGGAGACATGCAAAGGCTGTGCAGAAAAGTCTGACTAC       480
Ec      AAGAAGATCCTGGATGACTGGGGGGAGACGTGCAAAGGCTGTGCAGAGAAGTCTGACTAC       471
Bt      AAGAAGATCCTGGACGACTGGGGGGAGACGTGCAAAGGCTGTGCGGAAAAGTCTGACTAC       471
Ss      AAGAAGATCCTAGACGACTGGGGGGAGACGTGCAAAGGCTGTGCAGAAAAGTCTGACTAC       471
        ********  *  * ******** **    *** **** **    ** **    ** * *
```

```
Tc      ATCCAAAGAATAGAAGAATTGAAGCATAAACATACCGAACTTTAA>                    507
Hs      ATCCGGAAGATAAATGAACTGATGCCTAAATATGCCCCCAAGGCAGCCAGTGCACGGACC      540
Ec      ATCCGGAAGATTAATGAACTGATGCCTAAATACGCCCCTAAGGCAGCCAGTTCCCGGACT      531
Bt      ATCCGGAAGATTAATGAACTGATGCCTAAATATGCTCCCAAGGCAGCTAGTTCACGGACT      531
Ss      ATCCGGAAGATTAATGAACTGATGCCAAAATATGCCCCCAAGGCAGCCAGTTCACGGACT      531
        ****  *  **  * *** *** **  *** *  *         *

Tc                  507
Hs      GATTTGTAG>  549
Ec      GATTTGTAG>  540
Bt      GATTTGTAG>  540
Ss      GATTTGTAG>  540
```

	E. callabus	*H. sapiens*	*S. scrofa*	*T. castaneum*
B. taurus	0.069	0.071	0.057	0.547
E. callabus		0.071	0.063	0.598
H. sapiens			0.069	0.595
S. scrofa				0.573

References

Apostolou, A., Shen, Y., Liang, Y., Luo, J., & Fang, S. (2008). Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. *Experimental cell research*, *314*(13), 2454-2467.

Arakane, Y., Li, B., Muthukrishnan, S., Beeman, R. W., Kramer, K. J., & Park, Y. (2008). Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle,< i> Tribolium castaneum</i>. *Mechanisms of development*, *125*(11), 984-995.

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., & Kramer, K. J. (2005). Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. *PNAS*, *102*(32), 11337-11342.

Bennett, S. "Tribolium castaneum." Mathematics at the University of Texas at Austin: Mathematical Biology. 2003. 17 Apr. 2014. <http://www.ma.utexas.edu/users/davis/375/LECTURES/L2/Tribolium>.

Berghammer, A. J., Klingler, M., & Wimmer, E. A. (1999). Genetic techniques: a universal marker for transgenic insects. *Nature*, *402*(6760), 370-371.

Calvin, D. "Confused Flour Beetle and Red Flour Beetle." Penn State College of Agricultural Sciences Entomology. Nov. 1990. 17 Apr. 2014. <http://ento.psu.edu/extension/factsheets/flour-beetle>.

Cao, S. S., & Kaufman, R. J. (2012). Unfolded protein response. *Current Biology*, *22*(16), R622-R626.

Chaudhari, S. S., Arakane, Y., Specht, C. A., Moussian, B., Boyle, D. L., Park, Y., Kramer, K. J., Beeman, R. W., & Muthukrishnan, S. (2011). Knickkopf protein protects and organizes chitin in the newly synthesized insect exoskeleton. *PNAS*, *108*(41), 17028-17033.

Evron, E., Cairns, P., Halachmi, N., Ahrendt, S. A., Reed, A. L., & Sidransky, D. (1997). Normal polymorphism in the incomplete trinucleotide repeat of the arginine-rich protein gene. *Cancer research*, *57*(14), 2888-2889.

Heerman, M. (2014). *Analysis of EST'S Encoding Pea Aphid Acyrthosiphon pisum C002 & The Effect of Armet Transcript Knockdown in Tribolium castaneum* (Master's thesis, Kansas State University).

Hellman, M., Arumäe, U., Yu, L. Y., Lindholm, P., Peränen, J., Saarma, M., & Permi, P. (2011). Mesencephalic astrocyte-derived neurotrophic factor (MANF) has a unique mechanism to rescue apoptotic neurons. *Journal of biological chemistry*, *286*(4), 2675-2680.

Hoseki, J., Sasakawa, H., Yamaguchi, Y., Maeda, M., Kubota, H., Kato, K., & Nagata, K. (2010). Solution structure and dynamics of mouse ARMET. *FEBS letters*, *584*(8), 1536-1542.

Keeley, L. "Insect Cuticle-Structure and Molting." Physiology and Biochemistry Animations for Entomology and Biology Education. 2004. 21 Apr. 2014. <http://www.physioviva.com/movies/integument_structure/>.

Lorenzen, M. D., Berghammer, A. J., Brown, S. J., Denell, R. E., Klingler, M., & Beeman, R. W. (2003). piggyBac-mediated germline transformation in the beetle Tribolium castaneum. *Insect molecular biology*, *12*(5), 433-440.

Meyer, J. "External Anatomy The Exoskeleton." General Entomology NC State University. 2005. 21 Apr. 2014. <http://www.cals.ncsu.edu/course/ent425/tutorial/integ.html>.

Mizobuchi, N., Hoseki, J., Kubota, H., Toyokuni, S., Nozaki, J. I., Naitoh, M., Koizumi, A., & Nagata, K. (2006). ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. *Cell structure and function*, *32*(1), 41-50.

Palgi, M., Lindström, R., Peränen, J., Piepponen, T. P., Saarma, M., & Heino, T. I. (2009). Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. *PNAS*, *106*(7), 2429-2434.

Parkash, V., Lindholm, P., Peränen, J., Kalkkinen, N., Oksanen, E., Saarma, M., Leppänen, V-M., & Goldman, A. (2009). The structure of the conserved neurotrophic factors MANF and CDNF explains why they are bifunctional. *Protein Engineering Design and Selection*, *22*(4), 233-241.

Petrova, P. S., Raibekas, A., Pevsner, J., Vigo, N., Anafi, M., Moore, M. K., Peaire, A. E., Shridhar, V., Smith, D. I., Kelly, J., Durocher, Y., & Commissiong, J. W. (2003). MANF. *Journal of Molecular Neuroscience*, *20*(2), 173-187.

Schwarz, H., & Moussian, B. (2007). Electron microscopic and genetic dissection of arthropod cuticle differentiation. *Modern research and educational topics in microscopy Tuebingen: Formatex. p316-25*.

Shridhar, R., Shridhar, V., Rivard, S., Siegfried, J. M., Pietraszkiewicz, H., Ensley, J., Pauley, R., Grignon, D., Sakr, W., Miller, O. J., & Smith, D. I. (1996). Mutations in the arginine-rich protein gene, in lung, breast, and prostate cancers, and in squamous cell carcinoma of the head and neck. *Cancer research*, *56*(24), 5576-5578.

Shridhar, V., Rivard, S., Wang, X., Shridhar, R., Paisley, C., Mullins, C., Beirnat, L., Dugan, M., Sarkar, F., Miller, O. J., Vaitkevicius, V. K., & Smith, D. I. (1997). Mutations in the arginine-rich protein gene (ARP) in pancreatic cancer. *Oncogene*, *14*(18).

Tomoyasu, Y., & Denell, R. E. (2004). Larval RNAi in Tribolium (Coleoptera) for analyzing adult development. *Development genes and evolution*, *214*(11), 575-578.

Wilson, R. C., & Doudna, J. A. (2013). Molecular mechanisms of RNA interference. *Annual review of biophysics*, *42*, 217-239.

Zhu, X. (2011). *Intracellular localization, biochemical and biophysical properties of human Armet* (Master's thesis, Kansas State University).