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GENE EXPRESSION AND DEVELOPMENT IN
BLOWFLIES:
A METHOD TO MORE PRECISELY ESTIMATE
POSTMORTEM INTERVALS

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Kimberly C. Jennings

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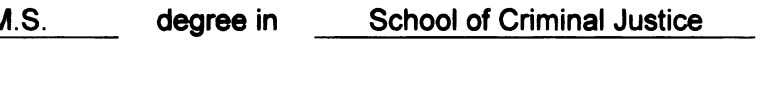
of the requirements for the

M.S. degree in School of Criminal Justice



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GENE EXPRESSION AND DEVELOPMENT IN BLOWFLIES: A METHOD TO MORE PRECISELY ESTIMATE POSTMORTEM INTERVALS

By

Kimberly C. Jennings

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

School of Criminal Justice

2007

ABSTRACT

GENE EXPRESSION AND DEVELOPMENT IN BLOWFLIES: A METHOD TO MORE PRECISELY ESTIMATE POSTMORTEM INTERVALS

By

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The use of insects in forensic entomology has become one of the most helpful tools for estimating post mortem interval (PMI). Within minutes of death, Calliphoridae flies colonize human corpses and are the most accurate to estimate PMI. Currently, length measurements of larvae combined with environmental temperature, as well as the number of posterior spiracles present are used to estimate the developmental stage of maggots. There are many drawbacks to using these methods for PMI determination, including shrinkage of larvae as the third instar ends and variation among growth rates. Therefore, alternative methods are needed to increase the precision of PMI estimation. Molecular studies, especially those including *Drosophila melanogaster*, have helped scientists understand gene expression and regulation. Throughout the life cycle of a fly, thousands of genes are up- and down-regulated, thus if one could determine the level at which a gene is expressed it may provide an estimation of age. The third instar is the longest and most difficult stage in *Lucilia sericata* to age, therefore ideal for gene expression analysis. Six genes were examined in the insect including ADP/ATP translocase, cuticle 1, chitin synthase, chymotrypsinogen, alpha trypsin, and aminopeptidase. Based on the research presented here, they show unique patterns that can be used to estimate larval development.

This thesis is dedicated to Tyler Williams, Timothy "T.J." Upshaw, and Justin "B.J." Passarge whose untimely deaths made me realize how valuable life is and what is truly important.

ACKNOWLEDGMENTS

I would like to thank my committee members for their reading and critique of this work, Dr. David Foran, Dr. David Arnosti, and Dr. Christopher Smith. Also to Aaron Tarone: thank you for your time and effort in helping me pursue this degree. I could not have done this without you, so thank you for being my 2nd "advisor"! I would like to acknowledge all of the current and past members of our laboratory for their encouraging words, long study nights, and support over the years. The friendships we have developed will last a lifetime. I also would like to thank the School of Criminal Justice for their financial support over the last 2 years.

Finally, my deepest gratitude to my family and friends for being a constant support system in my life, whether it was cheering me on at an athletic event, a school function, or life in general. Specifically, I want to recognize my parents who were my biggest supporters even in the most frustrating times and who over the years have always believed in me and encouraged me to pursue my dreams regardless of what anyone else thought; my brother Orville and sister Ashley for being my main motivation to achieve more, to want more, not just in school but in life as a whole; and Celina Green, whose friendship has extended far beyond that of just sorority sisters, thank you for always listening to me. These individuals have molded and shaped me into the person I am today and for that I am truly grateful.

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Introduction

Forensic Science, Entomology, and Postmortem Interval

Forensic science is defined as the application of science to the law. When human remains are discovered, there are four key questions asked: how, when, where, and why did that person die (Slone et al. 2005). Each question becomes especially important if suspicious circumstances surround the death, which in turn makes forensic scientists key figures in determining the answer. To establish when a person died, forensic scientists need to establish the postmortem interval or PMI— the time between the death of the victim and the discovery of the corpse. Time of death is important in criminal investigations because it helps create a time line of the victim's actions. An established PMI can also aid in the investigation by narrowing the list of suspects.

One of the most widely used methods to estimate PMI is analyzing decomposition in the body. Decomposition is the gradual breakdown of dead organic matter (Spitz 1993), which begins moments after death and continues over a period of time. This process can be divided into five stages: fresh, bloat, active decay, post decay, and dry (Figure 1) (Catts and Goff 1992). Each stage corresponds to a particular time range, for instance, the fresh stage lasts up to 3 days following death while the active decay stage can last between 10 and 20 days postmortem. Therefore, if the decompositional stage of the body can be determined, then a time of death can be roughly estimated.

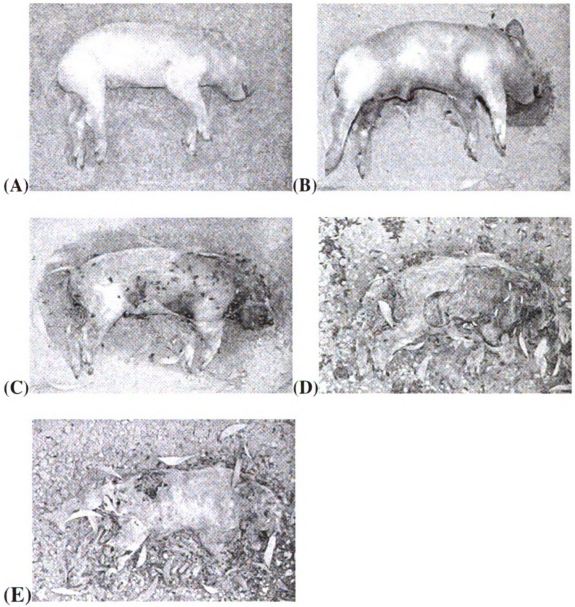

Figure 1. An illustration of the five stages of decomposition shown in a pig. (A) The fresh stage can last for up to 3 days following death. (B) The bloat stage can occur between 4 to 10 days. (C) The active decay stage can last anywhere from 10 to 20 days. (D) The post decay stage can last between 20 and 50 days. (E) The dry stage occurs after approximately 50 or more days. These stages of decomposition along with the correlated time frames are used to estimate PMI. However, the rate of decomposition can change based upon the ambient temperature, the environment the body is in, and the condition of the body. Photographs:
http://www.deathonline.net/decomposition/decomposition/index.htm

Various factors, such as rigor mortis, algor mortis, and chemical and physiological changes, can also be used to estimate PMI; however, as the PMI increases, the estimated range becomes wider (Spitz 1993). Rigor mortis, often called stiffness of death, is caused by a decrease in the production of ATP as well as an increasing acidity in the muscles after death making portions of the body stiff and unmovable. The process tends to follow a particular time line beginning between 2 and 4 hours post mortem. After 12 to 24 hours, full rigor has set in and over the next 12 to 48 hours, it will subside (Spitz 1993). Algor mortis, the gradual cooling of the body after death, is most helpful during the first 10–12 hours postmortem, assuming an ambient temperature of approximately 75°F and a normal healthy victim. The chemical and physiological changes that can be used to estimate PMI include looking at the changes in the level of potassium in the vitreous humour of the eye, as well as analyzing the stomach contents of the individual (Jackson and Jackson 2004, Spitz 1993). The use of decompositional stages, rigor mortis, algor mortis, and chemical or physiological changes to estimate PMI are often unreliable because of a high rate of variability. External factors, such as higher or lower ambient temperature, age of the deceased, body mass of the deceased, and the surroundings the body is in, can influence the time frame (Jackson and Jackson 2004, Strachan and Read 2004, Spitz 1993). Therefore, alternative methods need to be explored that would increase the precision of PMI estimation and in this regard, forensic entomology has become a helpful tool (Anderson 2005).

Forensic entomology is the use of insects and other arthropods to aid in legal investigations (Benecke 2001, Catts and Goff 1992). The field includes three categories:

1) stored or commercial product entomology, such as food contamination; 2) urban entomology, or how insects affect man and his environment, such as termites feeding on a house; and 3) medico-legal (medico-criminal) entomology, which focuses on the criminal component, such as homicides, suicides, and other violent felonious crimes (Spitz 1993). Benecke (2001) reported that the earliest known account of entomology in a forensic sense was in the 13th century by Sung Tzu. A man was stabbed near a rice field and his wounds were identified as having come from a sharp object, possibly a sickle used by the workers. The death investigator told all the workers to line up and lay out their sickles. Although the killer had wiped the blade to where no visible trace of tissue was present, enough blood remained on the sickle to attract flies. The owner of the sickle then confessed to the murder.

An important figure in the advancement of forensic entomology was Bergeret d'Arbois, a French doctor. In 1855 he looked at blowfly pupae and moths to estimate the PMI of a mummified child that had been found in the wall of a house in Paris (Benecke 2001, Catts and Goff 1992). Time of death needed to be determined to clear the couple that currently lived in the house. Bergeret described the life cycle of the insects he found from egg to adult, and estimated that the child had been dead for at least two years. His mistake, however, was assuming that the insects' metamorphosis from egg to adulthood took a full year to complete and this oversight distorted his PMI estimation (Benecke 2001, Greenberg 1991).

The forensically important insects (i.e. those associated with remains) can be placed into four categories: necrophages (feed on the tissue of the deceased) such as certain Diptera (flies) and Silphidae (carrion beetles), parasites/predators (feed/live on or

within other insects attracted to the corpse) such as certain Diptera or Silphidae, omnivores (feed on the corpse and other insects) such as Hymenoptera (wasps or bees), and incidentals (use the corpse for reasons other then feeding) such as spiders and butterflies (Campobasso et al. 2001, Catts and Goff 1992, Keh 1985). Necrophages, more specifically blowflies (Calliphoridae), are usually the basis for determining PMI because they are often the first to colonize human corpses, arriving minutes after death (Catts and Goff 1992). Adult females prefer to lay eggs (oviposition) in the wounds and orifices of a body because newly hatched larvae cannot break skin barriers; these locations on the decomposing body allow access to a liquid protein food source which is essential for their development, as well as providing a moist and humid environment that enhances survival (Ames and Turner 2003, Spitz 1993). Blowflies are not the only insects to colonize decaying remains; others include flesh flies (Diptera: Sarcophagidae), carrion beetles (Coleoptera: Silphidae), rove beetles (Coleoptera: Staphylinidae), as well as others (Campobasso et al. 2001).

PMI estimation is generally accomplished in one of three ways. The first is to survey the species found on the body and the time that is required for species to reach those specific stages of development, assuming that the insect begin colonizing the body soon after death (Catts and Goff 1992). Maggot length and environmental temperatures are often used to develop growth charts and thresholds, or the value that must be surpassed to begin producing a given effect or result (Donovan et al. 2006, Greenberg 1991). Since insects are poikilothermic (cold blooded), the rate of growth is proportional to the ambient temperature and fairly predictable. By knowing the length of the maggot and the ambient temperature its age can be estimated. For example, Greenberg (1991)

determined that if a green bottle fly (*Phaenicia (Lucilia) sericata*) was approximately 13mm in length and the environmental temperature was 22°C, then the maggot was 4 days old. There are, however, lower and upper thresholds to the growth rate, which are defined as the lowest temperature where development will occur and the minimum upper temperature where the rate of growth or development begins to decrease or stop, respectively (Figure 2). PMI can also be estimated by comparing the types of insects found on remains to the expected pattern of colonization. During the course of decomposition the assemblage of insects is fairly specific and reproducible (Catts and Goff 1992). However, the third and preferable way to estimate PMI is to use a combination of the previous two methods.

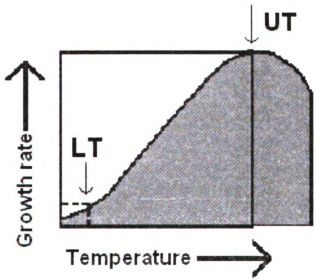

Figure 2. Diagram of typical insect development. LT stands for lower threshold, which is the minimum temperature where development occurs, and UT stands for upper threshold, which is the minimum upper temperature where the rate of growth or development begins to decrease or stop. As the ambient temperature increases so does the growth rate of the insect.

Development of Flies

L. sericata is a member of the Calliphoridae and an important insect in forensic entomology. It undergoes holometabolous, or complete metamorphosis, in which the young does not resemble an adult and the insect completes specific life stages, in this case: egg, larva, pupa, and adult (Figure 3).

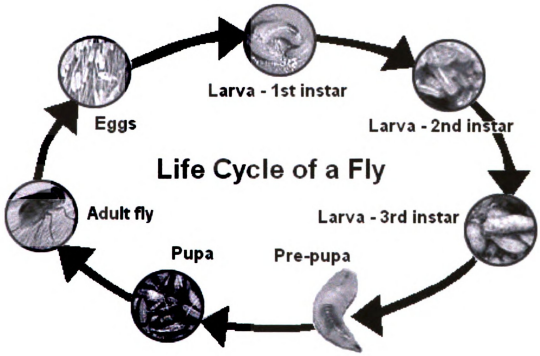

Figure 3. Life cycle of *L. sericata*. *L. sericata*, as well as other dipterids, undergoes holometabolous metamorphosis, in which the insect completes four specific life stages: egg, larva, pupa, and adult. The larval stage is divided into 3 separate instars (1st, 2nd, and 3rd) as well as the pre-pupa (post-feeding) stage. Image: http://www.susq-town.org/evans/forensic_sc_final_exam_rev2006Answers.htm

The life cycle begins with oviposition and upon hatching, the first instar stage commences. Since *L. sericata* has an exoskeleton, the insect can only grow to the size of the "shell". Once the larva reaches its maximum size, molting, or the shedding of the old cuticle (the outer layer of the body wall) and replacement with a new one, occurs. The molting of the cuticle signals the end of the first instar and the larva enters the second instar where it continues to feed and grow until molting occurs again, signaling the beginning of the third instar. This stage is unique in that it is divided into two sections: the feeding stage and a pre-pupa (post-feeding) stage, where the larva begins to consume the contents of its crop (food-storage organ in the foregut) for nutrients (Ames and Turner 2003). The first, second, and third instars can be distinguished from each other by larval length and the number of posterior spiracles (breathing tubes on the posterior end of the maggot). Following the end of the feeding stage, maggots migrate away from the food source. They also experience a reduction in larval size and the hardening of the outer skin in preparation for pupation, the gradual process of transforming larva into the adult form. The larva remains in the puparium (the protective case formed during pupation) until the adult is ready to emerge. At a constant temperature of approximately 20°C, development of egg to adulthood lasts approximately 2 weeks (Table 1).

Stage	Time in hours (Total time in hours)
Time between oviposition & eclosion	21 (21)
1st Instar	30 (51)
2nd Instar	55 (106)
3rd Instar	274 (380)
Pupation	244 (624)

Table 1. Typical blowfly development at a constant temperature of 20°C. The average time that blowflies spend in each developmental stage, except adult, is listed (Byrd and Allen 2001). The number in parentheses is the total time of development from oviposition.

Problems Associated with Using Entomology for PMI Estimates

While forensic entomology is an effective way to estimate PMI, it should be noted that it has some drawbacks. The figure that entomologists present for PMI is not an estimate of the time of death itself but rather the time between insect colonization and corpse discovery. The time frame assumes that insects will contact the body moments after death, thus if there are delays in the time it takes for that to occur the estimate will be incorrect. For instance, Rodriguez and Bass (1985) reported that the burial of a body, including depth and temperature, can affect insect colonization. Insect colonization in burnt bodies will also be delayed or limited (Spitz 1993). However, even though the colonization times differ between those with a delay and those without, insects will still find the remains, therefore each investigation must be examined on a case-by-case basis (Catts and Goff 1992).

As with any emerging science or technique, the *Daubert* standard should be met before being accepted by the courts. *Daubert v. Merrell Dow Pharmaceuticals*, 509 U.S. 579 (1993) changed the role of the courts in determining the relevancy and reliability of scientific evidence and the expertise of witnesses. The ruling states in order for evidence to be admissible in court: the theory must be tested; it must be subject to peer review and publication; the technique or method should have a known or potential error rate; and the technique must be generally accepted in the relevant scientific community. Even if evidence meets the requirements set forth by the courts through *Daubert*, it is not guaranteed that it will be admitted in the court of law. Evidence or an expert's testimony must also "assist the trier of fact to understand the evidence or to determine a fact in issue" (Federal Rules of Evidence, Rule 702). Forensic entomology is supported by a

large collection of literature that has been peer reviewed. However, when trying to estimate the developmental stage of larvae, variation among data sets is evident (Kaneshrajah and Turner 2004, Clark et al. 2006). This variation plays a crucial role when trying to use entomological evidence to estimate PMI because there is a lack of known error rates for the methods currently utilized in forensic entomology. To improve the precision of PMI estimation, methods need to be developed where error rates can be mathematically determined.

Variation in the growth rates of forensically important flies is obvious in many studies. For instance, Kaneshrajah and Turner (2004) reported that the growth rate of the blue bottle blowfly (*Calliphora vicina)* was different when reared on various food sources. They determined that *C. vicina* grew faster when feeding on lung, kidney, heart, or brain tissue than it did on liver. The rearing conditions also affected the developmental time required for larvae to reach each developmental stage, thus affecting the author's growth charts. Clark et al. (2006) found that *L. sericata* grew faster when reared on lung and heart then on liver. Larvae that fed on lung and heart entered the post-feeding stage approximately 31 hours earlier and were slightly longer in length when compared to liver fed larvae. These findings may have implications when estimating PMI in forensic cases because the location of the feeding larvae on the body may have an effect on developmental rate. These concerns were addressed by Tarone and Foran (2006) who showed that *L. sericata* development on rat carcasses was similar to flies reared on liver in a high moisture environment. The authors, using several treatment groups that consisted of providing cohorts with fresh liver every day or every third day, as well as a paper towel treatment in which a moistened paper towel was placed in the

bottom of a 1-liter canning jar, demonstrated that fly development times were significantly influenced by food moisture ($P<0.0001$). The paper towel treatment received fresh meat daily and length measurements were taken from each treatment group throughout the sample period. The authors reported that once the maggots stopped feeding moisture did not affect developmental time. This finding explains the conclusions of Kaneshrajah and Turner (2004) and Clark et al. (2006) which showed that fly development was delayed when reared on liver. Based on the results of the study by Tarone and Foran (2006) the delay that was noticed in the other experiments was due to the drying out of the liver.

The growth charts and thresholds that are experimentally used to estimate the age of maggots are produced at a constant temperature. However, since fly development depends largely on the ambient temperature, the fluctuating temperatures found in nature will have a major impact on insect development. One of the algorithms designed to take into account this variation is to measure accumulated degree days or hours (ADD or ADH). ADH or ADD is the temperature that the insect was reared, subtracted from the base temperature (which accounts for the positive temperatures where the insect does not develop), multiplied by time (Ames and Turner 2003). For example, if an insect was reared at 26.7°C for 16 hours and has a base temperature of 10°C its ADH is (26.7°C - 10°C) x 16 hours or 267.2 hours (Table 2). Any inaccuracies in the calculations, for instance using the wrong base temperature, could greatly alter the PMI estimate making this method very sensitive. Growth charts and thresholds can also be distorted by the persistence of development after the lower threshold has been reached. Since insects are influenced by the ambient temperature, theoretically once the threshold is reached

development will cease, however, Ames and Turner (2003) reported that development continued, at a reduced rate, in *C. vicina* and *Calliphora vomitoria* during a cold episode (10°C or less for five days). The continuation of larval development caused the ADH and ADD values to be distorted. If, however, remains were found in the winter and the body clearly showed the presence of insect activity, then it is reasonable for an entomologist to assume that death occurred during the prior period of warm weather.

Phormia regina—Rearing temperature 26.7°C				
B10-Base temperature of 10°C				
26.7°C-10°C=16.7°C				
16.7°C x Time (Hrs)=DH				
Stages	**Time (Hrs)**	**∑ Time (Hrs)**	**DH-B10**	**ADH-B10**
Eggs	16	16	267.2	267.2
1st Instar	18	34	300.6	567.8
2nd Instar	11	45	183.7	751.5
3rd Instar	36	81	601.2	1352.7
Prepupa	84	165	1402.8	2755.5
Pupa	144	309	2404.8	5160.3

Table 2. Development at a constant temperature of *Phormia regina*. The rate of development that Kamal (1958) estimated for *Phormia regina* showed that when reared at a constant temperature of 26.7°C, it took approximately 2 weeks to reach adulthood. From that degree hours (DH) and accumulated degree hours (ADH) can be calculated.

Gene Expression during Fly Development

The physical changes (for instance length) that a fly undergoes as it matures are not entirely accurate for estimating developmental age thus it is imperative that a more precise method to age larvae is developed. Molecular studies, especially involving

Drosophila melanogaster, have helped scientists better understand gene expression and regulation (Adams et al. 2000). Throughout the life cycle of a fly, genes are being up- and down-regulated, thus by determining the level of expression at a particular time genes can give a good estimation of developmental age.

Arbeitman et al. (2002) analyzed one-third of the *D. melanogaster* genome during each stage of development, from embryogenesis to adult. The authors reported that the expression level of many genes changed throughout the life cycle. For instance, there were two categories of transcripts present during embryogenesis, those that were produced from maternal genes and those that were expressed after fertilization. The transcripts with maternal origins were mostly degraded shortly after fertilization, whereas the ones which were zygotic increased in expression during the first 6–7 hours of development. The authors also looked at whether genes with similar functions were co-expressed during development and discovered that they were expressed at similar times, such as metabolic genes which were expressed at the highest level immediately before and during larval and adult life. In total, the authors reported that during embryogenesis 2,103 gene transcript levels changed out of the 4,028 analyzed; 445 changed during the larval stage, 646 changed during the pupal stage, and 118 changed during adult life.

Reverse Transcriptase and PCR

Reverse transcriptase, found in retroviruses, is a RNA-directed DNA polymerase. The enzyme can be used to make complementary DNA (cDNA) from mRNA, producing a DNA/RNA hybrid molecule (Strachan and Read 2004). The RNA strand is then

degraded and a second DNA strand complementary to the first is produced, creating a DNA/DNA molecule, whose quantity is proportional to the amount of starting mRNA.

The polymerase chain reaction (PCR) is a molecular technique used to exponentially amplify a specific region of DNA. This is a common method which when used with gel electrophoresis, or the separation of molecules via an electric charge, can quantify the amount of template present in a sample. By combining reverse transcription with PCR and gel electrophoresis, one is able to amplify and quantify RNA. To begin, RNA is isolated and reverse transcribed into its cDNA, after which standard PCR and gel electrophoresis can be initiated. Reverse transcription polymerase chain reaction (RT-PCR) is a sensitive technique for mRNA detection that allows for its amplification even in low copy number. RT-PCR can also be used to estimate a gene's expression level.

Study Aims

The feasibility of using gene expression levels to estimate the developmental age of third instar *L. sericata* was examined in this study. First RNA was isolated from larvae and quantified using RT-PCR and gel electrophoresis. Comparisons among time points between genes were used to estimate developmental age. Also examined during this study was the possibility of using a mitochondrial gene to identify the species of insect, which would eliminate the need to rear maggots to adulthood for species identification. Temperature and humidity are known to affect development time in *L. sericata* (Clark et al. 2006, Grassberger and Reiter 2001, Anderson 2000, Greenberg 1991) so these factors were kept constant to limit confounding variables within the experiment. The genes that were chosen for the project (listed below) were picked based

upon sequence availability (genes whose sequence was previously establish in *L. sericata, D. melanogaster,* or *L. cuprina*) and whether the expression level of the gene was likely to fluctuate during the third instar. The genes analyzed included:

ADP/ATP Translocase

ADP/ATP translocase is an abundant inner mitochondrial membrane protein (Louvi and Tsitilou 1992), which plays an important role in transporting ADP into the mitochondria and ATP into the cytoplasm and controlling the rate of oxidative phosphorylation. This gene was found to be expressed less as a cell ages (Fan et al. 1998), therefore the expression level is expected to decrease over time.

Alpha Trypsin

Trypsin is a major enzyme present in the gut during digestion that breaks down proteins (Barillas-Mury et al. 1995). Lemos et al. (1996) discovered that in mosquitoes, trypsin increases in an age-dependent manner before feeding. Therefore, examination of the feeding and post-feeding stages in *L. sericata* should show a difference in expression levels.

Aminopeptidase

Aminopeptidase is a metalloproteinase that cleaves amino acids from the carboxyl terminus of proteins and peptides (Reed et al. 1999). In *D. melanogaster* the major function of this enzyme is digestion of proteins, but it also has roles in nerve

transmission, tissue reorganization, and nutrient processing. A difference in expression levels is expected between the feeding and post-feeding stages of *L. sericata* larvae.

Chitin Synthase

Chitin synthase is the enzyme responsible for the formation of chitin, a major component of cuticular linings (lining of the foregut or hindgut of larvae) (Tellam et al. 2000). Chitin is a polymer that is extensively used in the exoskeleton of insects and cell walls. During *L. cuprina's* molting process, when a new exoskeleton is formed, a peak in the expression level of chitin was observed. Such an increase will be helpful in determining the end of an instar stage.

Chymotrypsinogen

Chymotrypsinogen is a precursor of chymotrypsin, which is a major excretory/secretory protease. Casu et. al. (1996) showed that enzyme is present in the gut of *L. cuprina* during each larval stage. This protease helps with the digestion process, thus once the maggot reaches the post-feeding stage a change in expression is expected (Reed et al. 1999, Casu et al. 1996).

Cuticle 1

Cuticle 1 is a cuticular protein, commonly found in the exoskeleton of insects. It can be a useful indicator of developmental progression in larvae because specific proteins are associated with certain stages of development (Snyder et al. 1982). Chihara et al. (1982) found that they could distinguish between a *D. melanogaster* "early" and "late"

third instar cuticle protein. The authors stated that cuticle 1 is considered a "late" protein that is secreted by the epidermal cells in the late third instar to help form the shell that will protect the developing larva throughout the pupal stage.

Cytochrome Oxidase 1

Cytochrome oxidase 1 is a mitochondrial gene that has been used to identify various Calliphoridae, as well as Sarcophagidae and other forensically important insects (Wells et al. 2001, Sperling et al. 1994). After amplification and sequencing of this region, comparisons can be made with published sequences to identify species. One advantage of using this gene over other methods for species identification, such as rearing larvae to adult, is that it allows for identification to occur even if the insect is no longer alive (Sperling et al. 1994).

Tubulin

Tubulin is a protein that polymerizes into long chains that form microtubules, which are the skeletal system for cells, essential for shape, division, and motility (Hutchens et al. 1997). Since tubulin is present at in all stages of development this gene can be used as a control in this experiment (O'Donnell et al. 1994).

MATERIALS AND METHODS

Fly Collection and Species Determination

In the fall of 2004, *L. sericata* adults were collected on the campus of Michigan State University in East Lansing, Michigan by Aaron Tarone. Species identification was completed by extracting DNA from three of the collected flies. Flies were placed in a 2mL tube along with 200µL of digestion buffer (20mM Tris, 50mM EDTA, 0.1% SDS) and ground with a tissue grinder, which was cleaned with 10 % bleach and rinsed with distilled water after each extraction. An additional 200µL of digestion buffer was added to the sample to complete grinding, and then incubated at 65°C for 30 minutes. Eight hundred µL of LiCl/KOAc (2.5 parts 6M: 1 part 5M) was added, and the sample was placed on ice for 15 minutes. The sample was centrifuged at 14,000 rpm (maximum speed) for 15 minutes at room temperature after which the supernatant was transferred to a new tube and 600µL of isopropanol was added. The tube was vortexed and centrifuged at maximum speed for 15 minutes at room temperature. The liquid was removed, making sure the pellet was not disturbed, and 100µL of 70% ethanol was added. The sample was centrifuged at maximum speed for 5 minutes, after which a majority of the liquid was removed with a pipette and then vacuum dried for 20 minutes. The pellet was resuspended in 150µL of TE buffer (10mM Tris, 1mM EDTA). The resuspended DNA was placed in a micron YM-100 column (Millipore) along with 300µL of TE buffer and centrifuged for 15 minutes at 500 x g. This step was repeated three times. The sample reservoir was placed upright over a clean vial and 100µL of TE was added. After pipetting up and down the reservoir was inverted over the new vial and centrifuged at 1000 x g for 3 minutes. The extract was then placed in a -20°C freezer.

The cytochrome oxidase 1 gene was PCR amplified and the sequence compared to sequences published on the NCBI website. The forward primers were 5'-taatattgctcatggaggag-3' and 5'-gatcagtagtaattacagct-3'; the reverse primers were 5'-cctaagaaatgttgagggaag-3' and 5'-ttgacttttaatatcttag-3', and the cycling parameters consisted of 2 minutes at 94°C, followed by 35 cycles of a 30 second 94°C denaturation step, a 1 minute 60°C annealing step, and a 1 minute 72°C extension step, then a final extension step of 5 minutes at 72°C. The samples were held at 4°C until removed from the thermocycler. The reaction was carried out in a total volume of 20μL, containing 1μL DNA template, 2μM primers, 0.2 mM dNTPs, 1X HotMaster Taq buffer (Eppendorf), and HotMaster Taq polymerase (Eppendorf) at 1U/reaction. Five μL of the reaction was loaded onto a 3% agarose gel and electrophoresed at 70 volts for approximately an hour. The gel was stained with ethidium bromide for 10 minutes and placed on a UV box to confirm amplification. The samples were placed in a Montage PCR Centrifugal Filter Device (Millipore) with 300μL of TE and centrifuged at 1000 x g for 15 minutes. This step was repeated making sure that all the TE was pushed through the filter. An additional 15μL of TE was placed on the filter and pipetted up and down. The reservoir was inverted over a new vial and spun at 1000 x g for 3 minutes. Samples were sequenced using a CEQ DTCS Quick Start Kit (Beckman Coulter) following the manufacturer's suggested protocol. The products were loaded onto a CEQ 8000 capillary electrophoresis instrument (Beckman Coulter), using the standard Long fast read (LFR-1) sequencing method (capillary temperature 50°C, denature 120 seconds at 90°C, inject 15 seconds at 2.0kV, and separate 85 minutes at 4.2kV) and aligned using BioEdit Sequence Alignment Editor (Hall 1999).

L. sericata adults were reared by A. Tarone. The cages of adult flies were given beef liver and checked approximately every 30 minutes until an egg mass (200–300 eggs) was laid, after which it was transferred to a clean glass jar, containing a moistened paper towel and approximately 4 ounces of fresh beef liver. The jar (group 1) was then covered with breathable cloth and placed into a 20°C incubator. This procedure was repeated with another egg mass (group 2). Approximately 4 ounces of fresh liver was added daily to each jar. First, second and third instar larvae were distinguished by the number of posterior spiracles. Once the cohort reached the third instar, collection began. Ten maggots were collected every 12 hours, of which three were randomly selected and placed in the appropriate sub-group based on this study's classification system (Figure 4). The maggots were stored in RNAlater (Ambion) at -80°C until RNA extraction.

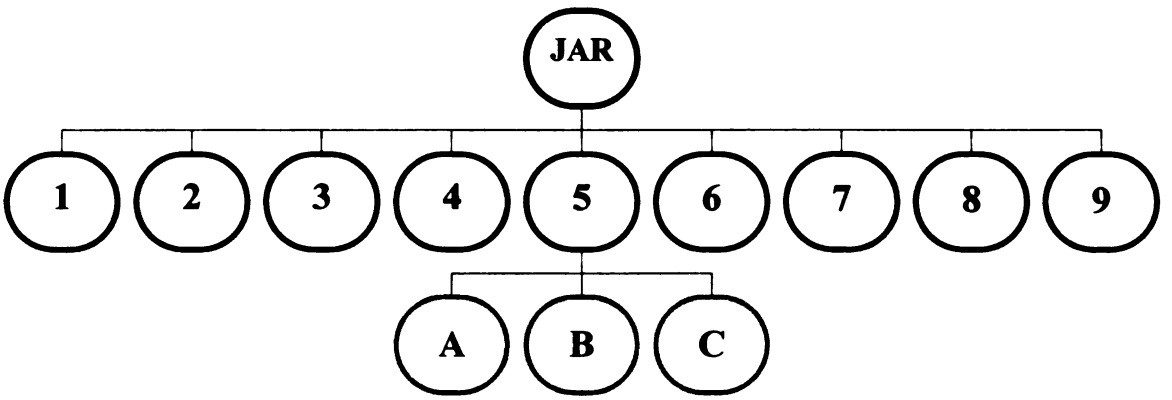

Figure 4. Diagram of how collection occurred. At the sampled time points 1–9, 10 maggots were collected. From these, 3 were randomly selected and placed into sub-groups (represented by A, B, and C). The diagram shows the sampling of a single time point; the same collection procedures were completed for each time point.

Primer Design

Genes for this project were selected based on their up- and down-regulation at various points throughout the third instar. Since there were few published gene sequences for *L. sericata*, sequences from related species were examined, including *L. cuprina* and *D. melanogaster*. A Blast (blastn) search was conducted on the NCBI website to find conserved regions for each gene. BLAST parameters were set to default values; *nr* for database (includes all GenBank, EMBL, DDBJ, PDB sequences but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences) low complexity filter, and Expect threshold = 10. From the sequence found on Blast, multiple primer pairs were designed using Primer 3 software (Rozen and Skaletsky 2000). The primer sets which produced sufficient product (i.e. intense brightness and little or no extra bands) were chosen for further analysis (Table 3).

Each gene was PCR amplified with the selected primers. The sequence obtained was inputted into the Blast search on the NCBI website to confirm identification (Table 3). The cycling parameters consisted of 2 minutes at 94°C, followed by 35 cycles of a 30 second 94°C denaturation step, a 1 minute 60°C annealing step, and a 1 minute 72°C extension step, then a 5 minute final extension step of 72°C. The samples were then held at 4°C until removed from the thermocycler. The reaction was carried out in a total volume of 20μL, containing 1μL DNA template, 2μM primers, 0.2 mM dNTPs, 1X HotMaster Taq buffer, and HotMaster Taq polymerase at 1U/reaction. Five μL of the reaction was loaded onto a 3% agarose gel and electrophoresed at 70 volts for approximately an hour. The gel was stained with ethidium bromide for 10 minutes and

placed on a UV box to confirm amplification. The samples were placed on a Montage PCR Centrifugal Filter Device (Millipore) with 300μL of TE and centrifuged at 1000 x g for 15 minutes. This step was repeated making sure that all of the TE was pushed through the filter. An additional 15μL of TE was placed on the filter and pipetted up and down. The reservoir was inverted over a new vial and spun at 1000 x g for 3 minutes. Samples were sequenced using the CEQ DTCS Quick Start Kit following the manufacturer's suggested protocol. The products were loaded onto the CEQ 8000 capillary electrophoresis instrument, using the program LFR-1 and aligned using BioEdit Sequence Alignment Editor (Hall 1999).

Gene	Primer Pair		Size
	Forward	Reverse	
ADP/ATP Translocase (ADP/ATP)	tcagccccgataagcaatac	tgttcttaggatcgggcaac	486
Alpha Trypsin (AT)	ttgttgtgcgctatttcctg	tcatgctgggtttgatttga	565
Aminopeptidase 1 (APN)	ttgtaccgctatgcgtgaag	tggaagatcatggcaatcaa	536
Chitin Synthase (CS)	gtacggctttgcagttaggc	tccacagaaccctcctcatc	364
Chymotrypsinogen (Chy)	caaatctgcttggtgtggtg	ggcacagacagcattgctaa	400
Cuticle 1 (Cut)	ttgctgctttgattgccttt	tcctttgagggatgagcttc	325
Cytochrome Oxidase 1 (CO1)	taatattgctcatggaggag gatcagtagtaattacagct	cctaagaaatgttgagggaag ttgacttttaatatcttag	776
Tubulin (Tub)	cgagacctactgcatcgaca	caccagatcgttcatgttgc	635

Table 3. The genes and primers chosen for analysis in *L. sericata*. The primer sequence of each gene used and the size of the expected amplicon are listed.

PCR Optimization

PCR parameters were optimized by examining four variables. Preliminary samples were tested with and without the addition of 1% formamide, with varying cycle number from 30 to 40, and with annealing temperatures of 55°C and 60°C. The various

forward and reverse primers that were designed for each gene were combined for PCR analysis and the set that showed a single band with the brightest intensity was chosen as the primer pair for that particular gene.

Gene Expression in L. sericata

RNA extraction was performed using a modified protocol from Whisenant (2006). Three maggots were placed in a 1.5mL tube and homogenized in 500µL of Trizol (Invitrogen) using a pestle that had been cleaned with 10 % bleach, rinsed with distilled water, and wiped with RNase*ZAP* (Ambion). The samples were incubated at room temperature for five minutes, then 200µL of chloroform was added and incubated for an additional three minutes at room temperature. The tubes were centrifuged at maximum speed for 30 minutes at 4°C. The aqueous layer was transferred to a new tube and 250µL of isopropanol was added. The samples were incubated for 10 minutes at room temperature and centrifuged at maximum speed for 30 minutes at 4°C. The supernatant was removed and the remaining pellet was washed with 1mL of 70% EtOH. The sample was spun at maximum speed for 15 minutes at 4°C. The pellet was vacuum dried for 20 minutes and resuspended in 60µL of RNAse free water (Ambion). Samples were stored at -80°C.

Seven µL of DNase I buffer and 7µL of DNase I enzyme (Ambion) were added to each sample and incubated for one hour at 37°C. The temperature was then increased to 75°C for 10 minutes. After the addition of 100µL of isopropanol, each tube was centrifuged at maximum speed for 30 minutes at 4°C. The liquid was removed and 60µL of 70% ethanol was added. Samples were centrifuged at 4°C for 30 minutes at maximum

speed. The supernatant was removed and the pellet was vacuumed dried. Thirty-two μL of RNAse-free water was added and the samples were stored at –80°C.

RNAs were quantified on a Beckman Spectrophotometer DU 520 General purpose UV/VIS single cell module using 1μL of sample added to 99μL of distilled H_2O. By dividing the 260 nm wavelength by the 280 nm wavelength, purity was calculated and the samples were diluted to 150ng/μL.

Reverse Transcription Reactions

RNA was reverse transcribed using an Applied Biosystems reverse transcription kit. A positive (+) RT reaction and negative (-) RT reaction were also prepared. The +RT reaction included 1μL of RNA, 1μL of reverse transcriptase (50 U/μL), and 18μL of premixed RT buffer, which contained MgCl, dNTPs, oligod(T), and RNase inhibitors. The -RT reaction contained 1μL of RNA and 19μL of RT buffer. Thermocycler settings were 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, and the samples were held at 4°C.

PCR Amplification of Genes

The PCR reaction contained 1μL cDNA, 2μM primers, 0.2mM dNTPs, 1x HotMaster Taq buffer, and HotMaster Taq polymerase at 1U/reaction in a total volume 10 μL. The program used to amplify each gene was comprised of a 2 minute 94°C initial denaturation step , followed by 40 cycles of a 30 second 94°C denaturation step, a 45 second 60°C annealing step, and a 1 minute 72°C extension step, and a 5 minute 72°C final extension step, afterwards the samples were held at 4°C. Five μL of the reaction

was loaded onto a 1% agarose gel and electrophoresed. The gel was stained with ethidium bromide and examined on a UV light box to compare band intensity of each time point.

Statistical Analysis

Bands on the gel were scored from 0 to 3, with 0 being no band present, 1 being a light band, 2 being a moderately intense band, and 3 being a bright, intense band. Tubulin was also scored for comparison purposes. If a tubulin sample was scored a zero, then that sample and all other samples made from the original dilution following RNA extraction, were not used for further analysis. If tubulin was scored as a light or moderate band then the other genes were analyzed accordingly. For instance, if tubulin received a call of 2 and alpha trypsin had a call of 1 for the same sample, then tubulin was adjusted so that its score would be a 3, causing alpha trypsin to be changed to a value of 2.

Statistical analysis of the data was conducted using one-way ANOVA in Microsoft Excel ($\alpha<0.05$). In order to tabulate the data, for every gene each time point was averaged and the means were all compared.

Results

Fly Collection and Species Determination

Amplification of DNA using cytochrome oxidase primers resulted in PCR products ranging from approximately 400bp to 450bp (Figure 5). Due to an inadequate cleansing step following PCR amplification, initial sequencing of the gene was unsuccessful. That was overcome by performing an additional 15 minute wash through a Montage PCR Centrifugal Filter Device. Sequencing produced a region of 774bp that was a 100% match to *L. sericata* (*P. sericata*) in the NCBI blast database, with a bit score of 1400–1421, thus confirming that the species was *L. sericata.* The bit score gives an indication of the alignment quality; hence the higher the score, the better the alignment. *L. cuprina* was the next closest match at 98%, with a bit score of 1376.

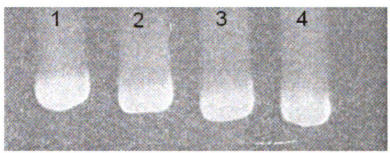

Figure 5. The amplification of DNA using cytochrome oxidase I primers. PCR amplification with cytochrome oxidase I primers produced sufficient product for sequencing. Lane 1, primers F3-R2, resulted in an amplicon that was approximately 450bp. Lane 2, primers F3-R, has an amplicon size of 400–425bp. Lane 3 has an amplicon size of approximately 400bp with the primer combination of F-R2. Lane 4 using primers F-R resulted in an amplicon of approximately 400bp.

Primer Design/PCR Optimization

Initially, the DNA was amplified using an annealing temperature of 60°C for 30 cycles. A majority of the reactions resulted in single, bright bands; however there were a few primer pairs that produced multiple products. The addition of formamide did not enhance the specificity of the reaction. The primer sets that produced a quality PCR product (i.e. little or no extra bands and intense brightness) following amplification were used for further analysis and sequenced to confirm gene identification (Table 3, Figure 6).

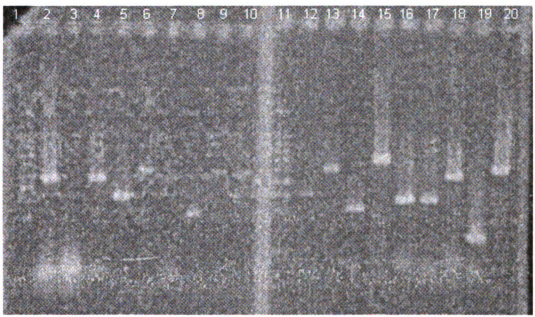

Figure 6. Example of optimization using various primer combinations. Amplification of extracted *L. sericata* DNA was done to determine the best primer combination for each gene analyzed throughout this study. Lanes 1 and 11 are 100 bp DNA ladders. Lanes 4–10 and 12 are the amplicons from APN. The primer pairs used were: Lane 4: F3-R3; Lane 5: F4-R4; Lane 6: F1-R3; Lane 7: F1-R4; Lane 8: F3-R1; Lane 9: F3-R4; Lane 10: F4-R1; Lane 12: F4-R3. Lanes 13–16 are from ADP/ATP and lanes 17–20 are primer combinations for AT. Lane 13: F1-R1; Lane 14: F2-R2; Lane 15: F1-R2; Lane 16: F2-R1; Lane 17: F1-R1; Lane 18: F2-R2; Lane 19: F1-R2; Lane 20: F2-R1. Primer combinations of F4-R1 (lane 10), F2-R1 (lane 16), and F1-R2 (lane 19) were chosen for further analysis in APN, ADP/ATP, and AT respectively.

+RT and –RT Reactions/PCR Amplification

The -RT samples using tubulin primers did not produce a DNA product (Figure 7), while amplification was seen in a majority of the samples containing reverse transcriptase using the tubulin primers (Figure 8). In general, the expression of tubulin was high throughout the feeding and post-feeding stages of the third instar. There were a few +RT samples that did not amplify, indicating that no cDNA was present, thus they were not included in the final analysis.

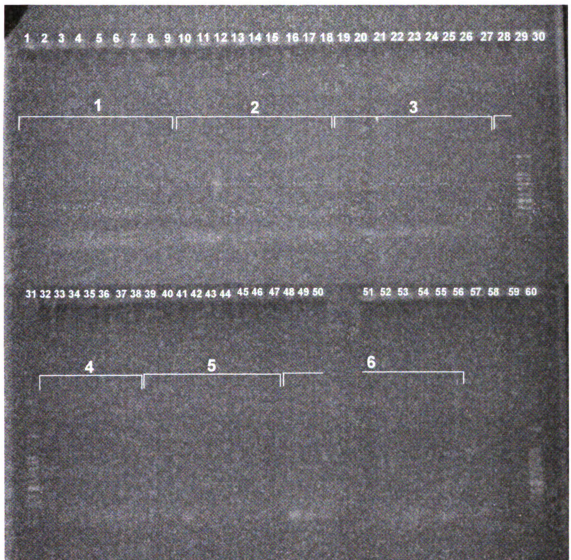

Figure 7. Amplification of the –RT samples. Amplification of –RT samples showed that no DNA contamination was present in the RNA extraction. Lanes 29, 31, and 60 are 100 bp DNA ladders. Lanes 1–28 correspond to samples 1–28 and lanes 32–59 correspond to samples 29–56. Brackets 1, 2, 3, 4, 5, and 6 corresponds to jar #1 subgroups A, B, and C and jar #2 subgroups A, B, and C, respectively.

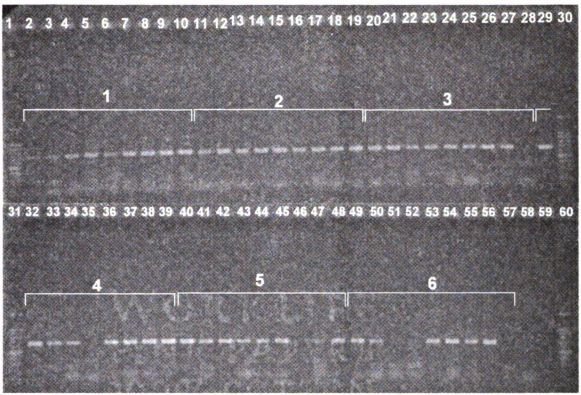

Figure 8. Amplification of the +RT samples. The samples shown in the photographs had bands of similar intensity. Lanes 1, 30, 31, and 60 are 100bp DNA ladders. Lanes 2–29 correspond to samples 1–28 and lanes 32–59 correspond to samples 29–56. Brackets 1, 2, 3, 4, 5, and 6 corresponds to jar #1 subgroups A, B, and C and jar #2 subgroups A, B, and C, respectively.

Statistical Analysis of Gene Expression

Based on the intensity of the bands, each was given a value between 0 and 3. The values for the time points were then averaged for each replicate (Table 4) and the mean was compared for each gene. For instance, during time point four the expression level of cuticle 1 was higher then the expression of aminopeptidase, while during time point nine aminopeptidase was higher then cuticle 1. The values were then used to statistically analyze the data and assemble graphs so the expression patterns could be easily visualized.

Gene	ADP/ATP	APN	AT	CS	CHY	CUT
Time Points						
1	2.83	0.83	0.50	1.40	0.17	2.17
2	2.67	0.83	1.33	2.00	0.83	2.83
3	2.60	0.80	1.80	2.20	0	2.80
4	2.80	1.00	1.40	2.20	0	3.00
5	2.83	2.17	1.67	0.80	0	2.50
6	3.00	1.00	1.33	1.00	0	2.00
7	3.00	1.00	0.60	0.20	0.40	1.60
8	2.40	1.20	0.20	1.00	0	1.40
9	3.00	2.25	0.75	1.50	0	1.50

Table 4. Gene expression values for each time point. The top row lists each gene used in the final analysis: ADP/ATP is ADP/ATP translocase, APN is aminopeptidase, AT represents alpha trypsin, CS corresponds to chitin synthase, CHY is chymotrypsinogen, and CUT is cuticle 1. The first column lists the experimentally defined time points which were 12 hours apart. The value within the table represents the average expression value from each subgroup.

ADP/ATP Translocase

Amplification of the ADP/ATP translocase transcript revealed that expression levels remained high throughout the feeding and post-feeding stages of the third instar (Figures 9 and 10). There was no significant difference in the expression level of the gene among the time points analyzed (P=0.84).

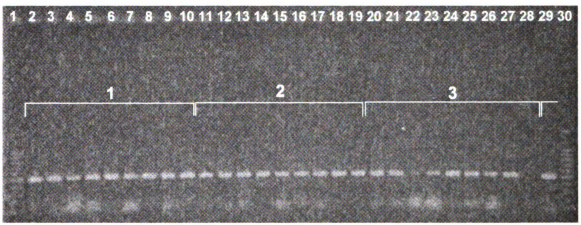

Figure 9. Amplification of the ADP/ATP translocase gene. Lanes 2–29 correspond to samples 1–28. Bracket number one (lanes 2–10) corresponds to sub-group A's nine time points; bracket number two (lanes 11–19) is sub-group B's nine time points; and bracket three (lanes 20–28) is sub-group C's nine time points (all from jar 1). Lane 29 is sub-group A for jar number 2. The sub-group follows a similar pattern to the others with samples being visually similar in intensity. Lane 28, which sample did not amplify, also failed to amplify when tubulin was analyzed. The bottom portion of the gel (which is not shown) does show some variation but it is very slight. The gene was not useful for the aging of *L. sericata.*

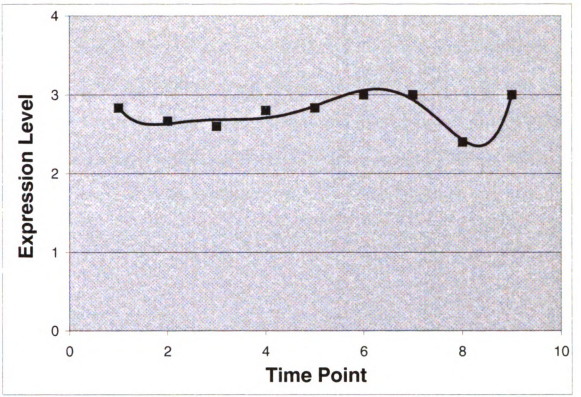

Figure 10. ADP/ATP translocase expression levels during development. The X-axis indicates the time point, while the Y-axis is the average level of expression. With the exception of a slight drop at time point eight, the level of expression in ADP/ATP translocase remained high throughout the third instar. When comparing the numerical values of time points eight and three they are similar.

Alpha Trypsin

The amplification of alpha trypsin mRNA showed a similar pattern among the samples analyzed. It appeared that during the beginning of the third instar the gene was expressed at low levels; however, during the middle of the third instar expression levels were higher. As the maggot entered the post-feeding stage (around collection point 5) expression levels lowered again (Figures 11 and 12). Overall there was no significant difference in the expression level of the gene among the time points analyzed (P=0.15).

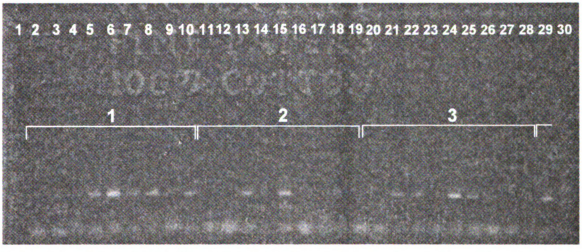

Figure 11. Amplification of the alpha trypsin gene. Lanes 2–29 correspond to samples 1–28. Bracket number one (lanes 2–10) corresponds to sub-group A's nine time points; bracket number two (lanes 11–19) is sub-group B's nine time points; and bracket three (lanes 20–28) is sub-group C's nine time points (all from jar 1). Lane 29 is sub-group A for jar number 2. Around collection point 5 (which correspond to lanes 6, 15, and 24) the maggots entered the post-feeding stage, where the highest level of expression was observed. Lane 28, which sample did not amplify also failed to amplify when tubulin was analyzed.

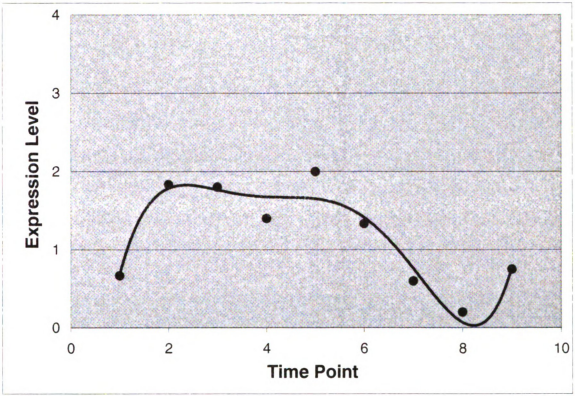

Figure 12. Alpha trypsin expression level during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. With the exception of time point one, expression of alpha trypsin remained at high levels until approximately the middle of the collection period (time point 5). Levels then remained fairly low for the remainder of the post feeding stage.

Aminopeptidase 1

PCR amplification of the aminopeptidase 1 transcript revealed a consistent expression pattern over most of the third instar. With the exception of time points 5 and 9, expression levels were low in this gene (Figure 13). Figure 14 shows the expression level of aminopeptidase, which did not vary significantly among the time points analyzed (P=0.15).

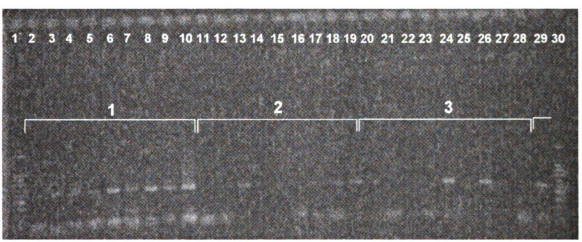

Figure 13. Amplification of the aminopeptidase 1 gene. Lanes 2–29 correspond to samples 1–28. Bracket number one (lanes 2–10) corresponds to sub-group A's nine time points; bracket number two (lanes 11–19) is sub-group B's nine time points; and bracket three (lanes 20–28) is sub-group C's nine time points (all from jar 1). Lane 29 is sub-group A for jar number 2. Each sub-group showed an increase in intensity over the third instar and post-feeding stages. Expression of aminopeptidase remained fairly constant with the exception of a few collection points, which on average showed brighter bands. Lane 28 which sample failed to amplify also did not amplify when tubulin was analyzed.

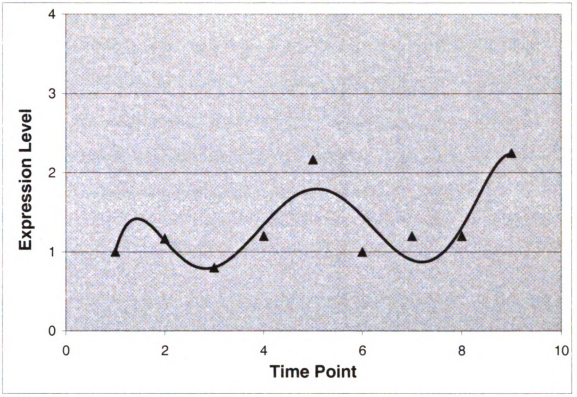

Figure 14. Aminopeptidase 1 expression level during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. Expression of aminopeptidase remained fairly constant with the exception of the 5th (beginning of post-feeding stage) and 9th (end of post-feeding stage) collection points.

Chitin Synthase

Amplification of the chitin synthase gene revealed that during the early third instar expression levels were fairly high. Expression then dropped to low levels during the middle and late third instar. However, right before pupation levels were higher (Figures 15 and 16). Statistical analysis confirmed that the expression level of chitin synthase was significantly different among the time points analyzed ($P<0.05$).

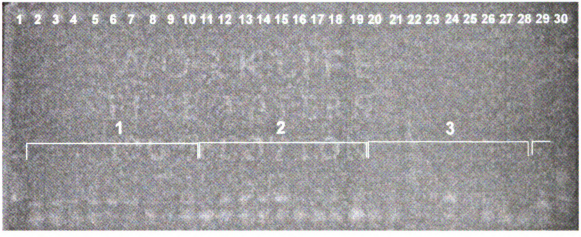

Figure 15. Amplification of the chitin synthase gene. Lanes 2–29 correspond to samples 1–28. Bracket number one (lanes 2–10) corresponds to sub-group A's nine time points; bracket number two (lanes 11–19) is sub-group B's nine time points; and bracket three (lanes 20–28) is sub-group C's nine time points (all from jar 1). Lane 29 is sub-group a for jar number 2. Expression of chitin synthase followed a similar pattern among each sub-group. The early collection points had on average higher levels of expression then the later collection points. Lane 28, which sample did not amplify also failed to amplify when tubulin was analyzed.

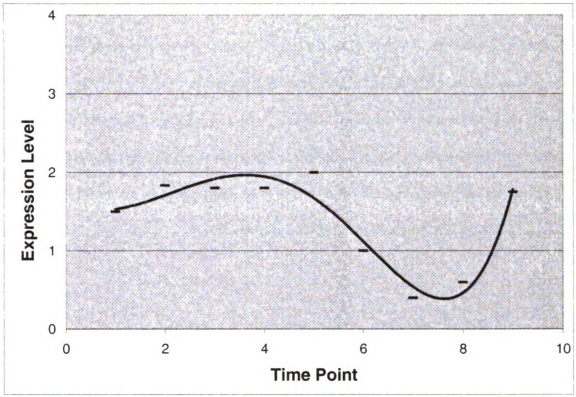

Figure 16. Chitin synthase expression level during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. Expression of the gene increased in intensity and remained high until shortly before the maggot entered the post-feeding stage. After a brief drop in expression, the level was slightly higher during time point nine leading into pupation.

Chymotrypsinogen

Amplification of the chymotrypsinogen transcript only occurred in a few of the samples (Figure 17). Since a majority of the samples did not amplify the amount of information obtained from this gene was not useful for statistical analysis (Figure 18). It is worth noting that during time points two and seven, a majority of the replicates had a light band. However, in lane 8 (time point seven, jar #1 sub-group A) and lane 26 (time point seven, jar #1 sub-group C) the band was slightly smaller then the expected size.

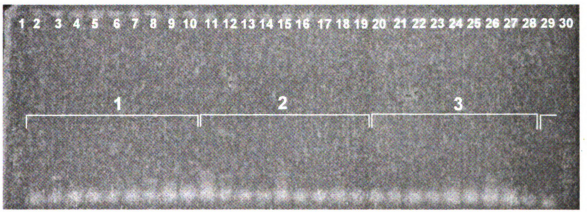

Figure 17. Amplification of the chymotrypsinogen gene. Lanes 2–29 correspond to samples 1–28. Bracket number one (lanes 2–10) corresponds to sub-group A's nine time points; bracket number two (lanes 11–19) is sub-group B's nine time points; and bracket three (lanes 20–28) is sub-group C's nine time points (all from jar 1). Lane 29 is sub-group a for jar number 2. Amplification of the chymotrypsinogen gene in many samples failed to occur, however, of the 6 replicates, 4 had a light band during the second time point. Three of the replicates had a band at time point seven, while time point one was not consistent, amplifying in only one replicate. Lane 28 which sample did not amplify also failed to amplify when tubulin was analyzed.

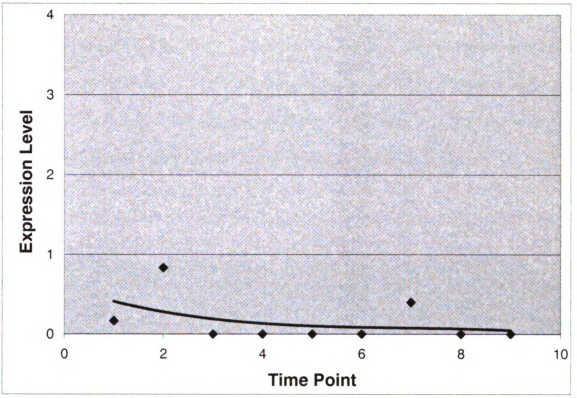

Figure 18. Chymotrypsinogen expression level during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. With the exception of time points one, two, and seven, expression of the gene was zero.

Cuticle 1

Amplification of the cuticle 1 transcript revealed that expression levels were high during the early third instar. After the feeding stage expression levels dropped until it reached its lowest level at time point eight (Figures 19 and 20). The expression level of the gene varied significantly between the time points analyzed ($P<0.05$).

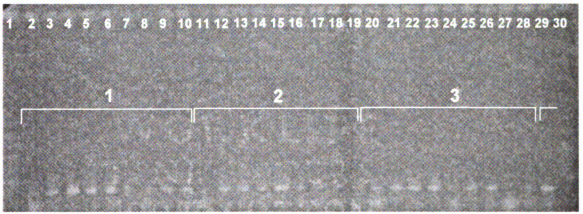

Figure 19. Amplification of the cuticle 1 gene. Lanes 2–29 correspond to samples 1–28. Bracket number one (lanes 2–10) corresponds to sub-group A's nine time points; bracket number two (lanes 11–19) is sub-group B's nine time points; and bracket three (lanes 20–28) is sub-group C's nine time points (all from jar 1). Lane 29 is sub-group A for jar number 2. The sample in lane 28 did amplify in this gene however due to the lack of amplification in tubulin it was not used for analysis.

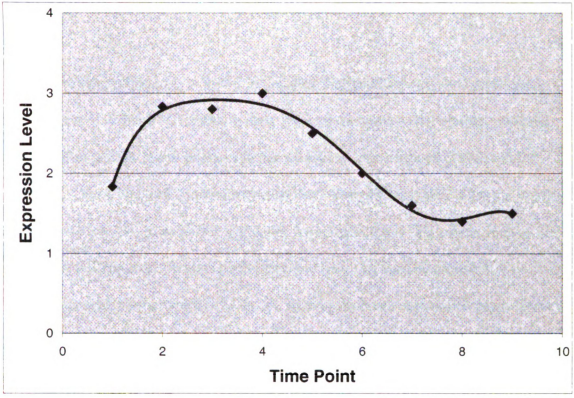

Figure 20. Cuticle 1 expression level during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. Cuticle 1was expressed at high levels during the beginning of the third instar followed by a steady decline until pupation occurred.

Discussion

Forensic entomology is useful in assisting with medico-legal investigations. Insects, more specifically Calliphorid flies, arrive at a corpse within minutes of death, thus based on the age of the larvae or pupae associated with a corpse, a PMI can be estimated. One of the main problems encountered when utilizing entomological evidence is the imprecision of age estimates during the longer developmental stages, such as the third instar and pupation. The research presented here was used to analyze RNA levels from a forensically important fly species, *L. sericata*, and investigate changes in gene expression that occur during the third instar, with the goal of using this information to increase the precision of PMI estimation.

Insects that are collected from a corpse during a death investigation must first be correctly identified since various species have different developmental rates. In many fly species of forensic importance, only subtle, phenotypic differences are found during the immature stages of development. Therefore, in many instances larvae must be reared to adulthood before accurate identification can be made, which is a difficult task that could also delay an investigation (Sperling et al. 1994). A few authors have demonstrated that by analyzing mtDNA sequences one could distinguish among members of the Sarcophagidae and Calliphoridae, (Wells et al. 2001, Sperling et al. 1994 respectively). A NCBI Blast search of 766 bp of cytochrome oxidase sequence obtained from blow flies utilized in the research presented here was a 100% match to *L. sericata* sequences in the database. The top 16 results were matches to *L. sericata* and all had a bit score of 1400–

1421, verifying species identification. *L. cuprina* was the next match in the database at 98%, with a bit score of 1376.

Gene Expression Analysis of L. sericata

The housekeeping gene tubulin was expressed throughout the third instar. The finding was consistent with those of O'Donnell et al. (1994) in which tubulin RNA was present in *D. melanogaster* during all stages of development and expressed at constant levels. In the research presented here, there were six samples in which amplification did not occur in the +RT reaction of tubulin, for example, the sample from jar #1, time point 9, sub-sample C (Figure 8; lane 28). Interestingly, in each instance where tubulin did not amplify, at least one of the other genes did show expression. However, these rare tubulin-negative results were not consistent across replicates, for instance, the only sample from jar #1, time point 9 that failed to amplify was the one displayed in lane 28. The results were also not consistent among extraction or amplification day, since each sub-group was prepared on a different day, indicating that the negative results could merely be a coincidence or the results of a failed PCR reaction. Regardless of their exact cause, the unusual tubulin-negative results most likely were due to technical errors, and thus not biologically meaningful. To prevent any discrepancies, if tubulin cDNA failed to amplify in any sample, that sample was excluded from further analysis.

Cuticle 1 and chitin synthase were the only genes whose expression levels varied significantly over the time frame analyzed. Cuticle 1 showed an increase in expression during the beginning of the third instar followed by a steady decline until pupation (Figure 20). Chihara et al. (1982) found that in *D. melanogaster* there were both early

and late third instar cuticle proteins. The early proteins were deposited in relatively large amounts during the feeding stage of the third instar, and had peak expression early; while the late cuticle proteins, which bind to chitin, were deposited after the larva finished feeding, when the puparium began formation. Charles et al. (1997) showed that *D. melanogaster* cuticle proteins have various conserved sequences grouped at position 65A on the third chromosome. Later Charles et al. (1998) re-examined the cluster, characterizing the proteins and expression patterns. They found that 10 cuticle proteins were expressed (LCP 1–10) during the third instar, six of which were classified as major proteins (1–6) and four as minor (7–10); cuticle 5, 6, and 8 were on cluster 65A; while 1–4 were encoded on the second chromosome clustered at 44D. The remaining three were either encoded at position 84A, also on the third chromosome, or at an unidentified location. No sequence similarity was found between the two clusters, but most of the proteins on 65A had reduced expression in the mid to late third instar coinciding with the pattern of a Chihara et al. (1982) defined early cuticle protein. The authors showed that three of the cuticle genes were roughly 70% similar (which they labeled e, f, and g), with three different developmental expression patterns. Two other genes, c and d, were 71% identical and followed similar but distinguishable expression patterns, while the b gene was expressed at high levels throughout the third instar until pupation when it disappeared. The a gene was not consistently expressed in the strains analyzed and the authors stated that further examination was necessary to determine its expression pattern. The cuticle sequence obtained during this experiment was an 82% match to *L. cuprina* cuticle 1 in Genbank; however, the expression pattern was completely different from that of a late cuticle. Based on the expression data obtained in the current study, the mRNA

examined was not from cuticle 1 but possibly from an early expression gene such as those encoded on cluster 65A of chromosome three.

Chitin synthase showed significant variation in expression levels throughout the third instar that could be used to help age larvae (Figure 16). Expression was highest during the early time points and lower during the end of the third instar. Various authors have analyzed the expression pattern of chitin synthase and found that between the third instar and pupation a change in the expression level occurred. Tellam et al. (2000) investigated the expression of chitin synthase in *L. cuprina* and showed that the level was slightly higher during the pupal stage then during the third instar. Gagou et al. (2002) found that in *D. melanogaster* third instar larvae the expression of chitin synthase was barely detectable, however, shortly after pupation commenced, levels increased. It is apparent from these studies that during the late third instar or beginning of pupation, an increase in the expression level of chitin synthase occurs. Zhu et al. (2002) analyzed the expression level of chitin synthase during late instar larvae and pupae of the tobacco hornworm (*Manduca sexta).* During feeding, transcript levels were relatively constant, but once feeding ceased expression dropped drastically before gradually increasing as the larva entered the pupal stage. This is why expression of the chitin synthase gene in the research presented here was higher during the beginning of the third instar (or the feeding stage) and then decreased until pupation. The result is consistent with the assumption that during morphological changes or when the insect's cuticle was altered in some way (such as molting), the expression level of chitin synthase changed. The changes can be helpful in determining the age of larvae because expression varies at specific points.

Another potentially informative gene throughout the third instar was alpha trypsin (Figure 12). Its expression was high during the early third instar and remained fairly consistent until time point five, after which it decreased before pupation. Lemos et al. (1996) found that in mosquitoes (*Anopheles gambiae*) trypsin mRNA accumulated in the tissues of newly emerged females before feeding, followed by a decrease in expression levels 36 hours after emergence. Since trypsin is one of the major enzymes required for digestion, expression would be expected to remain high during active feeding and decrease entering the post-feeding stage. The pattern observed in this study was consistent with the findings of others, and based on the data collected one can interpret that feeding ended around time point five. The change between the feeding and post-feeding stages of the third instar makes alpha trypsin an ideal locus for estimating development in *L. sericata.*

Three other loci, ADP/ATP translocase, chymotrypsinogen, and aminopeptidase did not show significant fluctuations in expression during the nine time points analyzed. ADP/ATP translocase levels were constant throughout most of the collection period, holding near an intensity of three—the highest level of expression, with the exception of time point eight which did have a lower level of expression (Figure 10). Louvi and Tsitilou (1992) showed that ADP/ATP translocase is an abundant protein of the inner mitochondrial membrane that plays an important role in transporting ADP into the mitochondria and ATP into the cytoplasm, thus gene expression would be obvious in all life stages. However, Fan et al. (1998) found that in human fibroblasts, ADP/ATP translocase was expressed less as a cell aged. This suggested that the expression level of the gene in *L. sericata* might also decrease over time, but it was not observed in the

research presented here. A decrease in expression could be undetectable in this experiment because the samples were from young maggots whose cells had not aged enough for a noticeable reduction in expression. Another possible reason why ADP/ATP translocase was not as useful might be that whole organisms were analyzed. In organisms cells are constantly being replaced and the rate of cell turnover could be too rapid for any decrease in expression to be observed. Therefore, ADP/ATP translocase can be a useful gene if older organisms were used and if they could be analyzed on a cellular level.

The major function of aminopeptidase is digestion of proteins (Reed et al. 1999). Expression of the locus was consistent around a level of 1, except at time points five and nine where it increased to level two (Figure 14). The increase at time point five coincides with the end of the feeding stage when the larva begins to consume the contents of its crop. The second expression increase (time point nine) marks the end of the post-feeding stage, when the larva undergoes multiple morphological changes in preparation for pupation. Therefore, based on the peaks in expression this research supports the hypothesis that aminopeptidase has a role in nutrient processing and tissue reorganization. The increases in expression observed, specifically at time points five and nine, indicate that this gene would be useful for estimating development during the third instar.

Chymotrypsinogen, the precursor of chymotrypsin, showed no expression during this experiment except during time points one, two, and seven (Figure 18). The level of expression in time point one was so low that simple stochastic effects might render it useless for this technique. Time point seven exhibited low levels of expression that were

consistent among replicates, while time point two showed an expression level of approximately 1, which was also constant among replicates. The variation in expression was not similar to the findings of Casu et al. (1996), where chymotrypsin was expressed at a moderate level during the third instar of *L. cuprina*. However, these authors gave little consideration to how long the maggots had been in the third instar, choosing to analyze whole stages only. Therefore, by random chance the authors may have chosen a maggot that had expression only during that particular time causing it to appear that expression occurred throughout the third instar. In this research the third instar was broken down into smaller segments thus if the maggot had expression at one time point and not another it would be detectable. This difference in sampling techniques could explain why the authors found the gene to be on whereas in the research presented here it was found to be off a majority of the time. Zhu et al. (2005) examined two groups of chymotrypsin genes in the Hessian fly (*Mayetiola destructor* [Say]). Group 1 contained six genes while group 2 had two genes, each being classified according to sequence similarity. Group 1 expression levels reached a maximum in four day old larvae, while group 2 genes were expressed in four and six day old larvae. Neither group was expressed in pupae or adults. Ramalho-Ortigao et al. (2003) also analyzed two chymotrypsin-like genes, which they labeled 1 and 2, in the sand fly (*Phlebotomus papatasi*). The first gene was expressed at very low levels in the midgut of non-fed insects, increasing sharply about 6 hours after feeding, then decreasing after 30 hours. The second gene behaved more as a "late chymotrypsin" in that it was first detectable 6 hours after feeding and increased until 30 hours. After 48 hours the expression of the second gene returned to undetectable levels. A common factor among these studies was

that expression of chymotrypsin did occur at certain points during the third instar, however, the discrepancy is the actual time it appears. Many factors could have caused the difference among findings. The authors either analyzed alternate forms of the chymotrypsinogen gene, which could have a slightly different expression pattern, or they analyzed the same gene in different species and the pattern varied among species. Regardless, the expression pattern of this gene warrants more investigation.

Combined Gene Expression Data and Analysis

By analyzing the expression information from ADP/ATP translocase, cuticle 1, chitin synthase, alpha trypsin, and aminopeptidase, the third instar can be divided into smaller segments. The combination of just two genes, cuticle 1 and chitin synthase, allows the division of the stage into two sections, early and late (Figure 21). Both loci were highly expressed had high levels of expression during the early portion of the third instar, while during the later half expression was low. Based on this pattern it is possible to estimate the age of larvae, for instance, if the expression level of cuticle 1 was high and chitin synthase was moderate then the larva would be 2 days into the third instar (Table 4). If the expression of cuticle 1 was moderate and chitin synthase was low then the larva would be roughly three days into the third instar, or at time point 6.

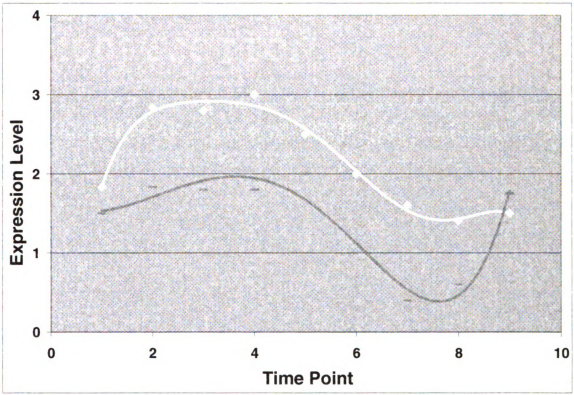

Figure 21. Expression level of chitin synthase (gray line) and cuticle 1 (white line) during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. Both loci had higher levels of expression early in the third instar but over time expression decreased. Using two genes it is possible to distinguish between early and late third instar larvae.

The third instar can be further separated into early, middle, and late portions by combining alpha trypsin and aminopeptidase expression data (Figure 22). During time point three the expression of aminopeptidase was at its lowest value while alpha trypsin was at its highest (Table 4); no other time point followed that pattern. The middle of the third instar can be differentiated based on the expression of aminopeptidase, which was above a level of two, while alpha trypsin was expressed at a moderate level. During time

point nine the expression level of aminopeptidase was also above a two; however, at that time alpha trypsin expression was low. The unique expression patterns of these genes could be used to distinguish early, middle, and late third instar larvae from each other.

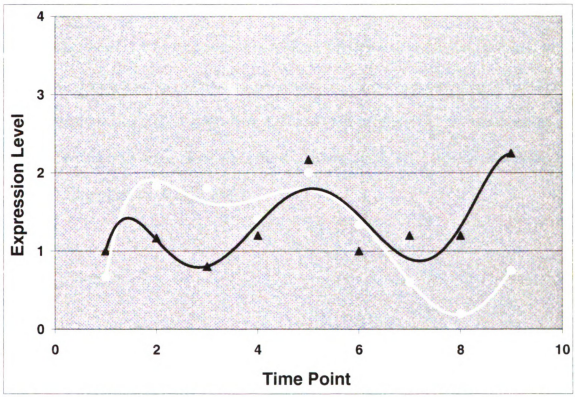

Figure 22. Expression levels of alpha trypsin (white line) and aminopeptidase (black line) during the third instar. The X-axis indicates the time point, while the Y-axis is the average level of expression. Alpha trypsin was expressed at higher levels during time points 2–5 while during the middle portion aminopeptidase was high. Later in the third instar alpha trypsin decreased in expression whereas aminopeptidase was high. Using these genes it is possible to separate the third instar into early, middle, and late sections.

By combining expression data from cuticle 1, chymotrypsinogen, chitin synthase, alpha trypsin, and aminopeptidase the third instar can be broken down into even more specific time points (Figure 23). For instance, during time point 2 chymotrypsinogen was

at its highest level while chitin synthase and alpha trypsin were at moderate levels. During time point 3, the expression levels of alpha trypsin and chitin synthase were at their highest values, 1.8 and 2.2 respectively, whereas aminopeptidase was at its lowest level of 0.80 (Table 4). Time point 4 can be distinguished by the levels of chitin synthase and cuticle 1, which were at their highest levels of 2.20 and 3.00. Cuticle 1 expression level during time point 6 was higher then both aminopeptidase and chitin synthase, while time point eight can be distinguished from the other time points because the expression level of cuticle 1 and alpha trypsin were 1.40 and 0.20, respectively. By combining the expression data from these genes all of the time points, with the exception of number 1, can be distinguished from each other.

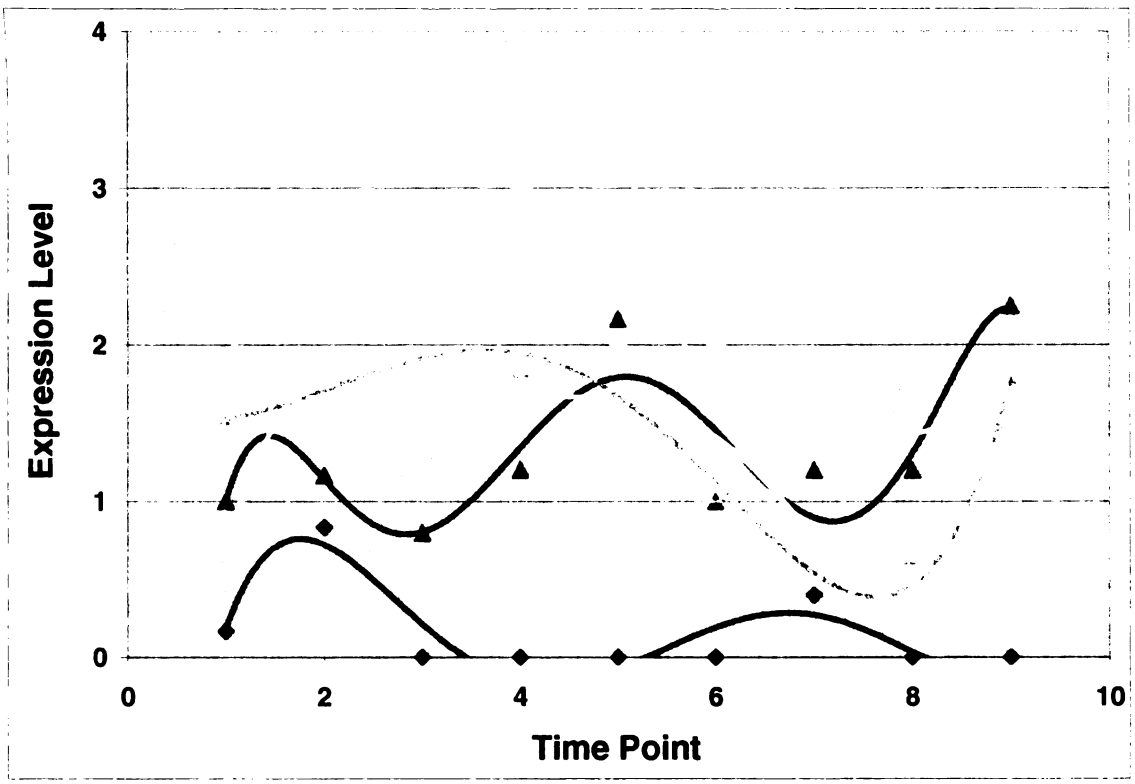

Figure 23. Expression levels of each gene during the third instar. The white line with diamonds represents cuticle 1, the gray line with dashes denotes chitin synthase, the white line with circles is alpha trypsin, and the black line with triangles represents aminopeptidase, while the black line with diamonds is chymotrypsinogen. The X-axis indicates the time point, while the Y-axis is the average level of expression. The combined expression data from each gene analyzed allows the separation of the third instar into specific time points. For instance, time points 2, 3, 4, 6, and 8 can all be distinguished based on the expression levels of each gene.

Experimental Error and Variation

In the research presented here, RNA levels of six genes were examined with the intention of developmentally aging third instar larvae. In doing so, it is important to understand the sources of error in this methodology and how to account for them. The amount of total error, or the difference between estimated or measured values and actual values within this experiment, depends on the degree of technical and biological 'noise'

(Deighton et al. 1978). Technical variation is a discrepancy in the procedure that is introduced during various sections of the experiment, and can be reduced by keeping each segment of the experiment as consistent as possible. On the other hand, biological variation is natural and influenced by genetic or environmental factors that are often beyond the control of the technician. However, there are certain steps that can be taken to identify error and help reduce noise so confidence intervals can be obtained.

Technical error has many potential sources. Any slight variation in the extraction of RNA, as well as the reverse transcription and PCR amplification steps, can cause the results of each trial to differ. To help minimize technical error, experimental controls (Winer 1971) were included that ensured the technique was working. Tubulin acted as the gene expression control in this experiment, as it is an essential protein for fly survival. There were some instances when tubulin mRNA did not amplify and the samples that produced negative tubulin results were excluded from analysis. A reagent blank was also prepared to confirm that reagents and supplies were free from DNA contamination that may have occurred during the extraction and amplification procedures, identifying false positives. To recognize technical error, replicate time points were tested in this experiment. For example, the sample in lane 28 of the tubulin gel was the only replicate of time point nine that showed no amplification (Figure 8). The negative product could have resulted from a technical problem during any part of the procedure. It does not appear that a bad reagent caused the negative result, however, as the lack of expression was not found in all the samples that were prepared using those reagents.

The method used to assign an expression level to each gene was another potential source of technical error. Band intensities for the time points were examined visually,

compared to the tubulin control gel, and assigned a numerical value. However, this method does not allow for the recognition of small differences in band intensity that may exist. An increase in the number of assigned values would potentially enhance the accuracy of the technique. For instance, using a 0 – 5 scale instead of the values 0 –3 would increase the resolution of the technique and allow more subtle differences among trials to be recorded. In this regard, using a visual method to assign expression levels numerical values has the potential to produce technical error. It is difficult for the eye to distinguish between small differences in band intensity, therefore as the range of values increases, potential error may also increase. Scanning and electronically analyzing the bands on the agarose gel would allow the intensity to be measured objectively. By taking into account the intensity of the control bands, the small differences among expression patterns could be recognized, minimizing error in this experiment.

Like technical error, biological error can also be recognized and accounted for. For instance, two maggots of the same age may show different expression levels for a particular gene. The variation observed may be caused by stochastic effects, which can be influenced by the number of cDNA copies that were put in the PCR tube. When the number of copies is extremely low each trial may contain different quantities thus by random chance amplification might be detected only in some cases. Also, if there are many copies of cDNA in each PCR tube, expression may appear to be intense in some trials and normal in others. As an example, time point one of the chymotrypsinogen gene had an amplicon that was visible in half of the replicates. The variation can be attributed to a small number of cDNA in each tube since amplification was only detected in a few instances.

In this experiment, several steps were taken to account for biological variability. To account for the inconsistency in gene expression levels among larvae, maggots were randomly placed into three sub-groups. This decreased the chance that the maggots chosen were from the same section of liver. It is possible that different regions of the food source had different nutritional qualities, influencing development. Assuring that the larvae selected for each replicate were from different locations, biological error was reduced. Sub-groups also contained three maggots and their mRNA was pooled for analysis rather then examining individuals separately. This helped to account for differences in expression resulting from individual variation, as well as increased the total amount of mRNA collected (Kendziorski et al. 2003). Pooling individuals allows the expression pattern common across a group to be analyzed instead of focusing on individual larvae. The effects of biological variation can also be minimized by testing replicate samples. For instance, larvae may develop at various rates, thus two maggots from the same cohort that hatched at similar times may be at slightly different points in development later in life, showing variability in gene expression. It was possible that some maggots were randomly chosen that had a faster/slower developmental rate than the other larvae, distorting the data. By replicating the experiment and averaging the data, any difference in expression that may arise from variation among samples is minimized, reducing biological variability.

Advantages of Using Gene Expression for PMI Estimates

The RNA based approach presented here is very appealing because error rates can be applied to the results which can be standardized. There are four Daubert criteria set

forth that would help judges determine whether a technique would be admissible during a trial. With the large collection of published literature and a general acceptance among the courts, the field of entomology currently meets most of the criteria. Error rates, a Daubert criteria, can be assigned to this technique because when using gene expression levels to determine the developmental age of larvae, a numerical value can be assigned to the expression pattern of a gene at a particular time point (Table 4). Aside from error rates, standard operating procedures (SOP) could also be developed for this method. With a variation in growth rates for species among many studies, a consistent technique needs to be developed that could help minimize the amount of technical error (Tarone and Foran 2006, Clark et al. 2006, Kaneshrajah and Turner 2004). SOPs will standardize a procedure ensuring that uniform techniques are used each time, improving the precision of PMI estimation.

Another advantage of using gene expression levels to estimate PMI is that the technique can be combined with real-time PCR or developed on a microarray to make estimating the developmental stage of larvae quicker and easier. Real-time PCR is a precise method that is used to quantify DNA and when combined with RT-PCR it can measure low abundance RNA. This would allow one to quantify gene expression at any particular time during the course of development. By placing all the useful genes on a microarray, including those for species identification, such as cytochrome oxidase, one could measure expression in a single experiment (NCBI 2004). This would be beneficial because as the use of gene expression becomes a widely accepted method for estimating PMI and more genes are added, analyzing one locus at a time becomes overwhelming

and less practical. Real-time PCR and microarrays would increase the convenience and rate at which these types of analyses can be conducted.

Considerations for Future Research

There were two factors that arose from this project that could be explored during future research: 1) increasing the number of genes analyzed and 2) tissue-specific expression pattern. An increase in the number of genes analyzed would help make the RNA-based technique more precise by allowing one to select informative genes, or genes that showed variability over the time frame investigated. Genes that have a definite on/off pattern at different times are ideal, whereas loci that show subtle changes over the development stage are less helpful. Loci that have roles in digestion, pupation, and instar transiting (molting), for example cuticle 1, chitin synthase, alpha trypsin, and aminopeptidase, are better suited for this type of analysis. These genes showed variation in expression levels that could be used to identify specific time points within the third instar. However, those genes that are essential for cell survival, such as ADP/ATP translocase, are not as useful because the level of expression stays fairly constant.

Another consideration for future research is the use of tissue-specific gene expression patterns to estimate the age of larvae. Tellam et al. (2000) found that during the third instar of *L. cuprina,* chitin synthase was expressed in a tissue-specific manner. There was no evidence of expression in the salivary gland, crop, cardia, midgut, or hindgut while the carcass and trachea showed amplification. If only a portion of a larvae was present on a corpse then an estimate of developmental age could still be determined based on the tissues present. Fyrberg et al. (1983) used RNA prepared from the heads,

thoraces, legs, and abdomens of late pupae *D. melanogaster,* as well as RNA from the ovaries of newly eclosed adults, to analyze levels of actin mRNA. The authors showed that the six actin genes (act88F, act5C, act42A, act57A, act87E, and act79B) were expressed at different levels in particular tissues at a specific time point. Act57A and act87E were present in the head and abdomen, act87E was found in the legs and ovaries, act88F and act79B were detected in the thorax, and act5C and act42A were not associated with any particular organ or muscle type. The expression data showed that one could estimate the age of developing larvae based on the expression pattern of the gene in a particular tissue, thus generating another way to estimate PMI.

Conclusion

There are many variables that need to be considered when using forensic entomology to help estimate a PMI. One must accurately identify the species of insect and determine its age before a PMI can be projected. While it is helpful to use other methods to age younger larvae, a more precise technique is needed for the longer-lasting third instar. Using the expression levels of genes to estimate the age of insects has the potential to greatly increase the accuracy of PMI estimation. The results of this study demonstrate that in *L. sericata*, gene expression levels could be used to developmentally age third instar larvae thus developing a more precise method to estimate PMI. The technique presented here has the potential to make estimating PMI, quicker, easier, and more precise.

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